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14. ABSTRACT

Androgen depletion therapy for advanced prostate cancer (PCa) invariably fails, and PCa recurs with an aggressive and lethal castration-resistant (CR) phenotype. Although androgen depletion inhibits activity of the androgen receptor (AR), continued AR function is important for resistance to androgen depletion. Therefore, even though this stage of the disease is often referred to as androgen-independent (AI), CRPCa remains an AR-dependent disease. Recently, alternatively-spliced, COOH-terminally truncated AR isoform variants have been identified in CRPCa cells and clinical tissues. These isoform variants function as constitutively active AR transcription factors that can support the CRPCa phenotype in model systems. The purpose of this project is to understand the mechanisms underlying increased expression and activity of these truncated AR isoform variants in CRPCa. Our work provides the first evidence that structural alterations in the AR gene may underlie disrupted AR splicing patterns at this stage of the disease. This knowledge could lead to better treatments or management of patients with CRPCa.

15. SUBJECT TERMS
None provided.

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INTRODUCTION

Androgen depletion therapy for advanced prostate cancer (PCa) invariably fails, and PCa recurs with an aggressive and lethal androgen-refractory or castration-resistant (CR) phenotype (also referred to as androgen-depletion-independent, abbreviated ADI). Although androgen depletion inhibits activity of the androgen receptor (AR), continued AR function is important for resistance to androgen depletion. Therefore, even though this stage of the disease is often referred to as androgen-independent (AI), CRPCa remains an AR-dependent disease. Recently, alternatively-spliced, COOH-terminally truncated AR isoform variants have been identified in CRPCa cells and clinical tissues [1-5]. These isoform variants function as constitutively active AR transcription factors that can support the CRPCa phenotype in model systems. The purpose of this project is to understand the mechanisms underlying increased expression and activity of these truncated AR isoform variants in CRPCa. The scope of the research is: 1. To study the structure of the AR gene and investigate whether intragenic rearrangements, including deletions and duplications, may underlie disrupted AR splicing patterns; and 2: to elucidate the biochemical properties of individual truncated AR isoform variants and determine whether different variants have differential capacities to support specific aspects of the CRPCa phenotype.

BODY

Findings resulting from TASK 1: Determine the locations of *AR* genomic break-points in 22Rv1 cells and AI LuCaP 35 xenografts

In our prior report, encompassing Year 1 of the award, we outlined the following research findings that resulted from completing project milestones:

1) The altered AR splicing patterns observed in the 22Rv1 cell line are linked to a 35kb tandem duplication encompassing AR exon 3 and flanking cryptic exons. We defined the nucleotide sequence of the break fusion junctions and demonstrated that this rearrangement explained the synthesis of the AR 1/2/3/2b mRNA. These findings are outlined in the attached publication (Li et al., *Cancer Research*, 2011) [6].
2) The CWR-R1 cell line, which also expresses high levels of truncated AR variants, harbors decreased copy number within the AR exon 1 region. This discovery was facilitated by our development of multiplex ligation-dependent probe assay (MLPA) as a new method to query AR copy number at distributed loci along the length of the gene.

In the current reporting period, we have made the following research findings as a result of completing the project milestones:

1) The preliminary data in Fig. 6A of our application indicated that the LuCaP 35 model of prostate cancer progression displayed unbalanced AR gene copy number when tumors progress to a CRPCa stage. These data were based on a quantitative PCR-based assay that we had developed, which has since been replaced by MLPA. As outlined in Fig. 1C of our recent publication (Li et al., *Oncogene*, 2012) [7], MLPA failed to detect overt AR gene copy number imbalances in CRPCa LuCaP 35 xenografts.
2) As a result of this negative finding in LuCaP 35, we shifted our focus to characterizing the intron 1 copy number loss in CWR-R1 in more detail. As outlined in our recent

publication, (Li et al., *Oncogene*, 2012) [7], a subset of cells in the CWR-R1 cell line harbors a 48kb deletion within AR intron 1. We were successful in mapping the break fusion junction of this intragenic deletion which revealed a signature of non-homologous end joining (Fig. 5 of Li et al., *Oncogene*, 2012) [7]. Further, we demonstrated that this intragenic deletion marked the CRPCa sub-population in this heterogeneous cell line that expressed high levels of the truncated AR 1/2/3/CE3 variant and grew under androgen independent conditions (Fig. 7 of Li et al., *Oncogene*, 2012) [7].

3) During the funding period, the LuCaP 86.2 xenograft was identified as a model of CRPCa that expressed high levels of the truncated AR v567es variant arising from the splicing machinery skipping AR exons 5-7 [4]. Using MLPA, we found that the LuCaP 86.2 xenograft displayed reduced copy number of AR exons 5-7 (Fig. 1c of Li et al., *Oncogene*, 2012) [7]. We were successful in mapping the break fusion junction of this novel 8.5kb intragenic deletion of AR exons 5-7, which revealed a signature of non-homologous end joining (Fig. 2 of Li et al., *Oncogene*, 2012) [7].

All Task 1 studies were completed during the current reporting period.

Findings resulting from TASK 2: Determine whether unbalanced AR amplification occurs in ADI vs AD PCa

In our prior report encompassing Year 1 of the award, we indicated that these studies were ongoing.

In the current reporting period, we have made good progress and report the following research findings as a result of completing project milestones:

1) We used MLPA to assess AR gene copy number in androgen-dependent PCa cell lines, androgen-dependent PCa xenografts, and PCa prostatectomy specimens. Overall, these androgen-dependent PCa cells displayed one intact AR gene copy, with the exception of the LuCaP 35 xenograft, which displayed four balanced copies of the AR gene (Fig. 1 of Li et al., Oncogene, 2012) [7]. These data support the concept that AR gene copy number imbalance does not occur in hormone naïve PCa.
2) We also used MLPA to assess AR gene copy number in CRPCa cell lines, xenografts, and rapid autopsy specimens. Overall, we found frequent AR gene amplification and/or complex patterns of AR gene copy number imbalance. These data support the concept that AR gene copy number imbalances may be an important component of CRPCa progression.

Findings resulting from TASK 3: Determine the biochemical properties of individual truncated AR isoforms

In our prior report encompassing Year 1 of the award, we indicated that these studies were ongoing.

In the current reporting period, we have made the following research findings as a result of completing project milestones:

1) The AR NH2-terminal domain (NTD) and the DNA binding domain (DBD) represent the core requirement for truncated AR variants to support androgen-independent growth of the 22Rv1 cell line (Figs. 9D and E of Chan et al., *JBC*, 2012) [8]. This is also the core domain required for truncated AR variants to induce an androgen-independent growth phenotype in androgen-dependent LNCaP cells (Figs. 9A and B of Chan et al., *JBC*, 2012) [8].
2) The AR NTD/DBD core is sufficient for truncated AR variants to activate endogenous AR target genes (Fig. 8 of Chan et al., *JBC*, 2012) [8].

We have obtained U of M IACUC approval and USAMRMC Animal Care and Use Review Office (ACURO) approval for the mouse xenograft studies outlined in this Task and will complete these studies in Year 3.

Findings resulting from TASK 4: Test transcriptional activities of individual and paired truncated AR isoforms to identify the molecular basis for differential transcriptional activity

In our prior report encompassing Year 1 of the award, we indicated that these studies were ongoing.

In the current reporting period, we have made the following research findings as a result of completing project milestones:

1) The AR NTD/DBD core is sufficient for truncated AR variants to access the nucleus in prostate cancer cells. Certain truncated AR variants such as AR 1/2/3/CE3 display more efficient nuclear localization due to reconstitution of the canonical bipartite AR nuclear localization signal (NLS) by cryptic exon splicing, but this reconstituted NLS is not an important biochemical determinant of truncated AR variant activity (Fig. 1-4 and 7 of Chan et al., *JBC*, 2012) [8].

We have been optimizing the transfection conditions to obtain robust chromatin immunoprecipitation (ChIP) data. These studies are ongoing and will be completed in Year 3.

KEY RESEARCH ACCOMPLISHMENTS

- The first reports of structural alterations involving the AR gene in PCa as a mechanism for synthesis for truncated AR variants supporting the CRPCa phenotype (Li et al., *Cancer Research*, 2011, Li et al., *Oncogene*, 2012).

- Development of a novel MLPA assay for assessing AR gene structure in prostate cancer cell lines, xenografts, and tissue specimens (Figs. 1 and 2).

- The first report of unbalanced AR gene architectures in CRPCa.

- Identification that the canonical AR nuclear localization signal (NLS) is not required for truncated AR variants to access the nucleus, bind chromatin, and activate AR target genes in PCa cells (Chan et a., *JBC*, 2012).

REPORTABLE OUTCOMES

Manuscripts

Li, Y., M. Alsagabi, D. Fan, G.S. Bova, A.H. Tewfik, and S.M. Dehm, Intragenic rearrangement and altered RNA splicing of the androgen receptor in a cell-based model of prostate cancer progression. Cancer Res, 2011. 71(6): p. 2108-17.

Dehm SM, Tindall DJ. Alternatively spliced androgen receptor variants. *Endocrine Related Cancer*, 18:R183-196, 2011.

Li, Y., T.H. Hwang, L. Oseth, A. Hauge, R.L. Vessella, S.C. Schmechel, B. Hirsch, K.B. Beckman, K.A. Silverstein, and S.M. Dehm, AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression. *Oncogene*, 2012. In Press.

Chan, S.C., Y. Li, and S.M. Dehm, Androgen receptor splice variants activate AR target genes and support aberrant prostate cancer cell growth independent of the canonical AR nuclear localization signal. *J Biol Chem*, 2012. 287(23): p. 19736-49.

Presentations

NCI Special Conference: Androgen Receptor Signaling in Prostate Cancer: Translating Biology into Clinical Practice. December 6 & 7, 2010. Crystal City, Virginia. "Detection of Treatment-Dependent AR Gene Alterations".

Department of Defense Prostate Cancer Research Program Innovative Minds in Prostate Cancer Today (IMPaCT) Meeting, Game-Changing Research **Plenary Session**, March 9-11, 2011. Orlando, FL. "AR Intragenic Rearrangement and Altered mRNA Splicing in Castration-Resistant Prostate Cancer".

Society for Basic Urologic Research (SBUR) Spring Program at the American Urological Association (AUA) Annual Meeting, May 14, 2011. Washington, DC. "Androgen Receptor Gene Structure and Splicing Alterations in Prostate Cancer".

Prostate Cancer Foundation Scientific Retreat. September 22-24, 2011. Lake Tahoe, NV. "Intragenic *AR* Rearrangements and Splicing Alterations in Castration-Resistant Prostate Cancer".

American Association for Cancer Research (AACR) Special Conference: Advances in Prostate Cancer Research. February 6-9, 2012, Orlando. FL. "Altered AR gene architecture and splicing in castration-resistant prostate cancer".

Joint Research Seminar Series, Kimmel Cancer Center, Department of Cancer Biology, Thomas Jefferson University. March 29, 2012. "AR Genomic and Splicing Alterations Driving Prostate Cancer Progression".

Endocrine Society Annual Meeting (ENDO). Symposium Session: The Androgen Receptor: Eveready Battery of Prostate Cancer. June 23-26, 2012. Houston, TX. "Androgen Receptor Splice Variants in Prostate Cancer Progression".

Modulating Androgen Receptor Splice Variants in Castration-Resistant Prostate Cancer (MARS-CRPC). June 27, 2012. Dallas, TX. "AR Splice Variant Generation".

Grant Applications

RSG-12-031-01 Dehm (PI) 01/01/2012-12/31/2015
American Cancer Society
"AR Gene Structure Alterations and Prostate Cancer Progression"
Role: Principal Investigator
Status: FUNDED

R01CA174777 Dehm (PI) 04/01/2013-03/31/2018
"AR Gene Rearrangements and AR Signaling in Prostate Cancer
Role: Principal Investigator
Status: PENDING

SPORE in Prostate Cancer Tindall (PI) 09/01/2012-08/31/2017
"Role of the AR Amino Terminal Domain in Prostate Cancer Progression"
Role: Project 1 Leader
Status: PENDING

CONCLUSION

Rearrangements in the AR gene represent a new mechanism of CRPCa development and progression. Our work demonstrates that AR gene rearrangements drive splicing alterations favoring synthesis of truncated AR variants that can function as constitutive, ligand-independent AR transcription factors. These truncated AR variants are impervious to PCa therapies targeted to the AR ligand binding domain.

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APPENDICES

APPENDIX 1:
Li Y, Alsagabi M, Fan D, Bova GS, Tewfik AH, Dehm SM. Intragenic rearrangement and altered RNA splicing of the androgen receptor in a cell-based model of prostate cancer progression. *Cancer Res.,* 20:2108-17, 2011

APPENDIX 2:
Dehm SM, Tindall DJ. Alternatively spliced androgen receptor variants. *Endocrine Related Cancer*, 18:R183-196, 2011.

APPENDIX 3:
Li, Y., T.H. Hwang, L. Oseth, A. Hauge, R.L. Vessella, S.C. Schmechel, B. Hirsch, K.B. Beckman, K.A. Silverstein, and S.M. Dehm, AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression. *Oncogene,* 2012. In Press.

APPENDIX 4:
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Cancer Research

Intragenic Rearrangement and Altered RNA Splicing of the Androgen Receptor in a Cell-Based Model of Prostate Cancer Progression

Yingming Li, Majid Alsagabi, Danhua Fan, et al.

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Cancer Research

Intragenic Rearrangement and Altered RNA Splicing of the Androgen Receptor in a Cell-Based Model of Prostate Cancer Progression

Yingming Li[1], Majid Alsagabi[2], Danhua Fan[3], G. Steven Bova[4], Ahmed H. Tewfik[2], and Scott M. Dehm[1,5]

Abstract

Androgen depletion for advanced prostate cancer (PCa) targets activity of the androgen receptor (AR), a steroid receptor transcription factor required for PCa growth. The emergence of lethal castration-resistant PCa (CRPCa) is marked by aberrant reactivation of the AR despite ongoing androgen depletion. Recently, alternative splicing has been described as a mechanism giving rise to COOH-terminally truncated, constitutively active AR isoforms that can support the CRPCa phenotype. However, the pathologic origin of these truncated AR isoforms is unknown. The goal of this study was to investigate alterations in AR expression arising in a cell-based model of PCa progression driven by truncated AR isoform activity. We show that stable, high-level expression of truncated AR isoforms in 22Rv1 CRPCa cells is associated with intragenic rearrangement of an approximately 35-kb *AR* genomic segment harboring a cluster of previously described alternative AR exons. Analysis of genomic data from clinical specimens indicated that related *AR* intragenic copy number alterations occurred in CRPCa in the context of *AR* amplification. Cloning of the break fusion junction in 22Rv1 cells revealed long interspersed nuclear elements (LINE-1) flanking the rearranged segment and a DNA repair signature consistent with microhomology-mediated, break-induced replication. This rearrangement served as a marker for the emergence of a rare subpopulation of CRPCa cells expressing high levels of truncated AR isoforms during PCa progression *in vitro*. Together, these data provide the first report of *AR* intragenic rearrangements in CRPCa and an association with pathologic expression of truncated AR isoforms in a cell-based model of PCa progression. *Cancer Res; 71(6); 2108–17. ©2011 AACR.*

Introduction

Prostate cancer (PCa) is the most frequently diagnosed male cancer in the United States and the second leading cause of male cancer deaths (1). Normal prostate tissue requires androgens for healthy function and cellular homeostasis. Androgens exert their cellular action by binding to the androgen receptor (AR), a 110-kDa transcription factor and member of the steroid nuclear receptor family (2). Initially, PCa depends on normal androgenic activation of the AR for ongoing growth and survival and presents as an androgen- and AR-dependent disease. Therefore, androgen depletion is the standard systemic therapy for locally advanced or metastatic PCa (3). The limitation of androgen depletion is that PCa eventually recurs with a lethal, castration-resistant phenotype. Although this stage of the disease seems to be independent of normal androgenic signaling, it is well established that castration-resistant PCa (CRPCa) remains AR-dependent through various mechanisms of aberrant AR activation and the AR remains an important therapeutic target for CRPCa (4).

AR mutations, which can broaden AR ligand specificity, and *AR* amplification, which can lead to AR protein overexpression, are 2 genomic mechanisms that can support the CRPCa phenotype (5–14). Ligand-independent AR activation has also been described and can occur through enhanced dependence on mitogenic signaling cascades that converge on the AR and associated transcriptional coregulators (15). More recently, alternative splicing was described as a mechanism of aberrant AR activation in CRPCa (16–20). Wild-type AR is a modular protein with an NH_2-terminal (NTD) transcriptional activation function-1 (AF-1) domain, a central DNA-binding domain (DBD), and a dual-function COOH-terminal ligand-binding domain/AF-2 domain. Splicing of cryptic exons or exon skipping can yield truncated AR isoforms consisting of the NTD, DBD, and short, variable-length C-terminal extensions

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(16–20). These truncated AR isoforms are constitutively active and can support various features of the CRPCa phenotype such as the androgen-independent activation of *AR* target genes and androgen-independent growth. Importantly, truncated AR isoforms have been observed in various PCa cell lines, xenografts, and clinical samples, which supports an important role in disease progression (16–20).

Alternative splicing is a widespread mechanism for increasing diversity from a single gene (21), and normal regulation of this process is disrupted in pathologic conditions such as cancer (22). The discovery of alternatively spliced AR isoforms has underscored the importance of understanding how AR splicing may be disrupted in CRPCa. This could provide clues to how truncated AR isoforms play a pathologic role at later stages of the disease. Therefore, the purpose of this study was to investigate the mechanisms underlying changes in AR isoform expression in a cell-based model of PCa progression.

Materials and Methods

Cell culture

Benign prostate BPH-1 cells were generously provided by Dr. Haojie Huang (University of Minnesota) and cultured in RPMI 1640 (Invitrogen) with 10% FBS (Invitrogen). The CRPCa 22Rv1 cell line was obtained from American Type Culture Collection and cultured in RPMI 1640 medium with 10% FBS. Androgen-dependent PCa CWR22Pc cells were generously provided by Dr. Marja Nevalainen (Thomas Jefferson University; ref. 23) and cultured in RPMI 1640 supplemented with 10% FBS, 2.5 mmol/L L-glutamine, and 0.8 nmol/L dihydrotestosterone (DHT; Sigma). Cell growth in RPMI 1640 medium containing 10% charcoal-stripped serum (CSS) ± 1 nmol/L DHT was monitored by crystal violet staining. For androgen response experiments, cells were cultured in RPMI 1640 + 10% CSS for 48 hours, treated at $t = 0$ with 1 nmol/L DHT (Sigma) or vehicle (EtOH), and then harvested at indicated time points. For long-term androgen deprivation, 22Pc cells were cultured in RPMI 1640 + 10% CSS for 7 days and then split to fresh plates in RPMI 1640 + 10% CSS. Cells were trypsinized and reseeded in RPMI 1640 + 10% CSS after an additional 10 days to disperse emerging foci of growth. Samples were harvested following 7, 12, 17, 22, 27, and 32 days of culture in RPMI 1640 + 10% CSS.

Western blot

Western blotting of CWR22Pc and 22Rv1 lysates with AR (Santa Cruz N-20), ERK-2 (Santa Cruz D-2), and ARV-7 (Precision Antibody no. AG10008) antibodies was conducted exactly as described (16).

Quantitative real-time RT-PCR

Total cellular RNA was isolated from CWR22Pc and 22Rv1 cells as described (24). RNA was reverse transcribed using a RT kit and an oligo(dT) primer (Roche). Absolute quantitation of AR mRNA species was carried out using forward and reverse primers listed in Supplementary Table S1. Concurrently, quantitative PCR with serial dilutions of plasmids harboring wild-type AR, AR 1/2/2b, AR 1/2/3/2b, AR 1/2/3/CE1, AR 1/2/3/CE2, and AR 1/2/3/CE3 cDNAs was carried out using a SYBRGreen FastMix reaction cocktail (PerfeCTa; VWR Life Sciences) and an iCycler instrument (BioRad) exactly as described (16). Threshold cycle (C_t) of amplification values obtained from cDNA standards were used to construct C_t versus cDNA standard copy number standard curves. The values of C_t obtained from real-time reverse transcriptase PCR (RT-PCR) were plotted on these standard curves to derive copy number values for individual AR mRNA isoforms. For relative quantitation, fold expression change relative to glyceraldehyde 3-phosphate dehydrogenase was determined by the comparative C_t method ($2^{-\Delta\Delta C_t}$).

Genomic PCR

Genomic DNA was isolated from BPH-1, CWR22Pc, and 22Rv1 cells, using a NucleoSpin kit (Clontech). Genomic DNA from clinical CRPCa tissues was isolated as described previously (25). PCR primers were designed using the Primer3 program of the MacVector software package and are listed in Supplementary Table S1. For copy number determination, quantitative PCR with serial dilutions of BPH-1 genomic DNA was carried out for each primer pair, using SYBRGreen fastmix and an iCycler instrument. The values of C_t obtained from BPH-1 genomic DNA dilutions were used to construct C_t versus genomic copy number standard curves, with the inference that one BPH-1 genome contains one copy of the X chromosome and therefore one copy of the target region. The values of C_t obtained from test genomic DNA in real-time PCR reactions were plotted on these standard curves to derive genomic copy numbers for each of the PCR target regions. For conventional PCR, genomic DNA was amplified using a Taq Polymerase PCR kit (Qiagen), according to the manufacturer's protocol. For long-range PCR, genomic DNA was amplified using outward facing primers (Supplementary Table S1) and a LongRange PCR kit (Qiagen). Cloned PCR products originating from the *AR* locus were completely sequenced to identify the 22Rv1 *AR* locus break fusion junction.

Affymetrix Genome-Wide Human SNP Array 6.0 analysis

Affymetrix SNP6.0 profiling of primary PCa (26) and metastatic CRPCa (25) was done in previous studies. Raw data in .CEL format was obtained from the Gene Expression Omnnibus web site (accession numbers GSE18333 and GSE14996). Copy numbers were calculated for each probe set, using Partek Genomics Suite 6.4 analysis software with default settings. Briefly, for each probe set, raw intensity was corrected for fragment length and sequence and the geometric means of allele intensity values were scaled to 1 (0 in $\log_2$ space). Copy number was calculated from these summarized intensities by normalizing intensity of each individual tumor samples to the mean intensity of the pooled noncancerous samples. Probe-level copy number data were used as input in an algorithm designed to determine the collection of breakpoints that satisfy the maximum

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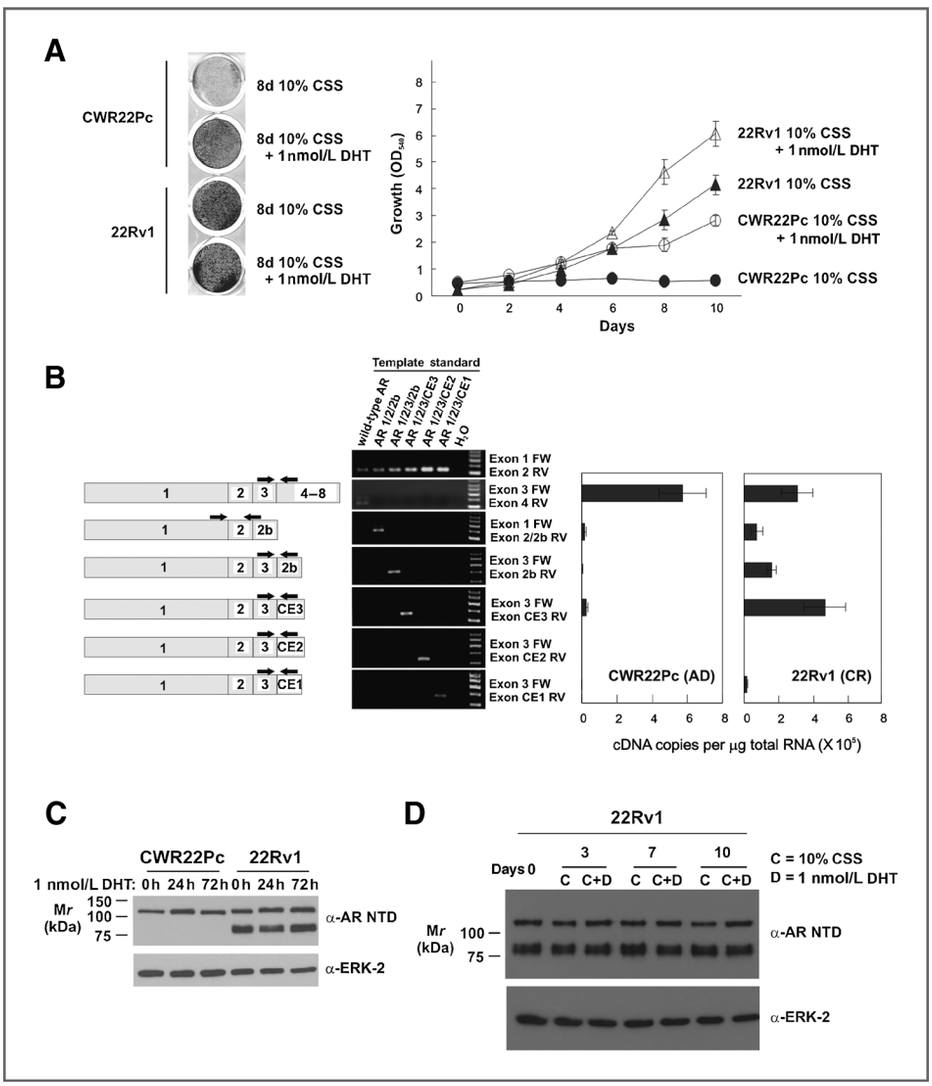

Figure 1. Efficient and stable synthesis of alternatively spliced *AR* mRNA isoforms in CRPCa cells. A, growth of CWR22Pc and 22Rv1 cells in the presence or absence of androgens. B, plasmid templates harboring depicted cDNAs were subjected to PCR with indicated primer pairs. Right, mRNAs from CWR22Pc and 22Rv1 cells were subjected to quantitative RT-PCR, using indicated primer sets. C_t values obtained from quantitative RT-PCR reactions were converted to copy number by plotting sample C_t values on C_t versus copy number standard curves constructed from concurrent quantitative PCR analysis of serial dilutions of plasmid templates. Data represent mean ± standard error from 2 independent experiments, each conducted in triplicate ($n = 6$). C, AR Western blots of CWR22Pc and 22Rv1 cells following 3-day treatment with 1 nmol/L DHT. ERK-2, loading control. D, AR Western blot of 22Rv1 cells following 10-day culture in steroid-depleted medium containing 1 nmol/L DHT or vehicle control. ERK-2, loading control.

likelihood between the input data and the noise-free version. The detailed algorithm is described in the Supplementary Methods section and is available in MATLAB (The MathWorks) upon request.

Results

AR intragenic rearrangement and aberrant *AR* mRNA splicing

The CWR22Pc cell line was recently established from the CWR22 human PCa xenograft (23). This cell line is androgen dependent for growth, which is in contrast to the CWR22-derived CRPCa 22Rv1 cell line (Fig. 1A; ref. 27). Recent reports have shown that alternatively spliced, truncated AR isoforms support constitutive AR-mediated transcription and androgen-independent proliferation of 22Rv1 cells (16–18). We therefore examined whether these isoforms were also synthesized in CWR22Pc cells. Using different PCR primer sets with different amplification efficiencies to identify the various *AR* mRNA isoforms precludes the use of the differential C_t of amplification ($2^{-\Delta\Delta C_t}$) method for determining relative expression by real-time PCR. We therefore pursued RT-PCR–based absolute quantification (Fig. 1B). As previously reported, full-length *AR* expression and high-level expression of the *AR* 1/2/2b, *AR* 1/2/3/2b, and *AR* 1/2/3/CE3 isoforms were observed at the mRNA and protein level in 22Rv1 cells (Fig. 1B and Supplementary Fig. S1). In androgen-dependent CWR22Pc cells, full-length *AR* expression was predominant but expression of AR 1/2/3/CE3 mRNA and protein was also detectable (Fig. 1B and Supplementary Fig. S2). No substantial change in these AR expression patterns was observed following 24 or 72 hours of treatment with androgens (Fig. 1C and

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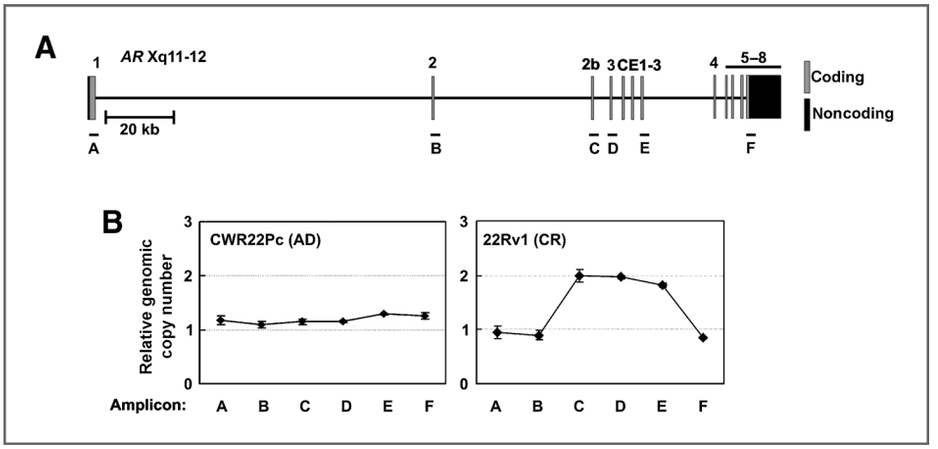

Figure 2. Alternatively spliced *AR* exons are contained on a rearranged genomic segment in 22Rv1 cells. A, schematic of the approximately 180-kb *AR* locus at Xq11-12. PCR amplicons used for copy number determination are labeled A to F. B, genomic DNA from CWR22Pc and 22Rv1 cells was subjected to quantitative PCR, using amplicon primer pairs indicated in A. C_t values were converted to copy number by plotting sample C_t values on C_t versus copy number standard curves constructed from serial dilutions of BPH-1 genomic DNA. Data represent mean ± standard error from 2 independent experiments, each conducted in triplicate ($n = 6$).

Supplementary Fig. S2). Similarly, AR isoform expression was stable during 10 days of 22Rv1 cell culture in the presence or absence of androgens (Fig. 1D). Together, these data show that both androgen-dependent CWR22Pc and CRPCa 22Rv1 cells can synthesize truncated AR isoforms but 22Rv1 cells can sustain stable, high-level expression.

Because the full-length *AR* mRNA in 22Rv1 cells has 2 copies of exon 3, resulting in an AR DBD with 3 zinc fingers (28), and because 22Rv1 cells can efficiently synthesize mRNAs with contiguously spliced exons 1, 2, 3, and 2b (16), we hypothesized that a genomic aberration in the 180-kb *AR* locus at Xp11-12 may underlie the stable splicing alterations observed in these cells. We therefore interrogated copy number at distributed loci along the length of the *AR* gene. Strikingly, in castration-resistant 22Rv1 cells, we observed increased copy number of AR exons 2b, 3, and CE3, suggesting a rearrangement involving this genomic segment (Fig. 2). This aberration was not observed in the androgen-dependent CWR22Pc cell line (Fig. 2). These data therefore suggest that alternative AR isoforms, which support the castration-resistant phenotype of 22Rv1 cells, may arise via enhanced splicing of alternative exons harbored on a rearranged genomic segment in the *AR* locus.

AR intragenic copy number alterations in metastatic CRPCa tissues

To determine whether *AR* intragenic copy number alterations occurred in human CRPCa, we analyzed high-resolution Affymetrix Genome-Wide Human SNP Array 6.0 (SNP6.0) data derived from clinical primary PCa ($n = 44$ tissues) from 44 patients and metastatic CRPCa ($n = 58$ tissues) from 14 patients (25, 26). To localize the boundaries of putative breakpoints, we used a dynamic program that estimates the number and locations of segments adaptively on the basis of probe-level data. This analysis revealed a high incidence of rearrangement in conjunction with *AR* amplification, only in CRPCa, which to our knowledge is a novel phenomenon that has not been described (Supplementary Fig. S3). Because the outcome of the 22Rv1 *AR* rearrangement seemed to be a focal copy number increase of a segment between *AR* exons 2 and 4, resulting in higher dosage of exon 3 and alternative exons relative to *AR* exon 4 (Fig. 2), we asked whether these phenomena occurred in clinical PCa. Indeed, focal copy number increases were observed between *AR* exons 2/3 and 3/4 in 12 of 58 (20.7%) metastases from 6 of 14 (42.9%) subjects, which presented as rearrangement of a segment harboring some or all alternative *AR* exons 2b or CE1-3 (Fig. 3). For most of these CRPCa samples, the outcome was the higher gene content of a segment containing *AR* exon 3 and alternative exons compared with *AR* exon 4 (Supplementary Fig. S4). These alterations were not observed in genomic DNA samples from these subjects' normal tissue (Supplementary Fig. S5). Focal copy number increase of this segment in a CRPCa subject was confirmed using targeted quantitative PCR (Supplementary Fig. S6). SNP6.0 analysis revealed no changes in overall *AR* copy number or focal alterations in this region in 44 primary PCa samples (Supplementary Fig. S4), indicating that CRPCa patients are more likely to harbor this rearrangement in at least one of their tumors than patients with localized, androgen-dependent PCa (6/14 vs. 0/44; $P = 0.000074$, Fisher's exact test). Overall, these data suggest that the region encompassing *AR* exon 3 may represent a "hotspot" for intragenic rearrangement in CRPCa.

AR breakpoint junction boundaries lie within LINE-1 elements in 22Rv1 cells

To establish with more precision the breakpoint junctions between *AR* exons 2 and 2b and *AR* exons CE3 and 4 in 22Rv1

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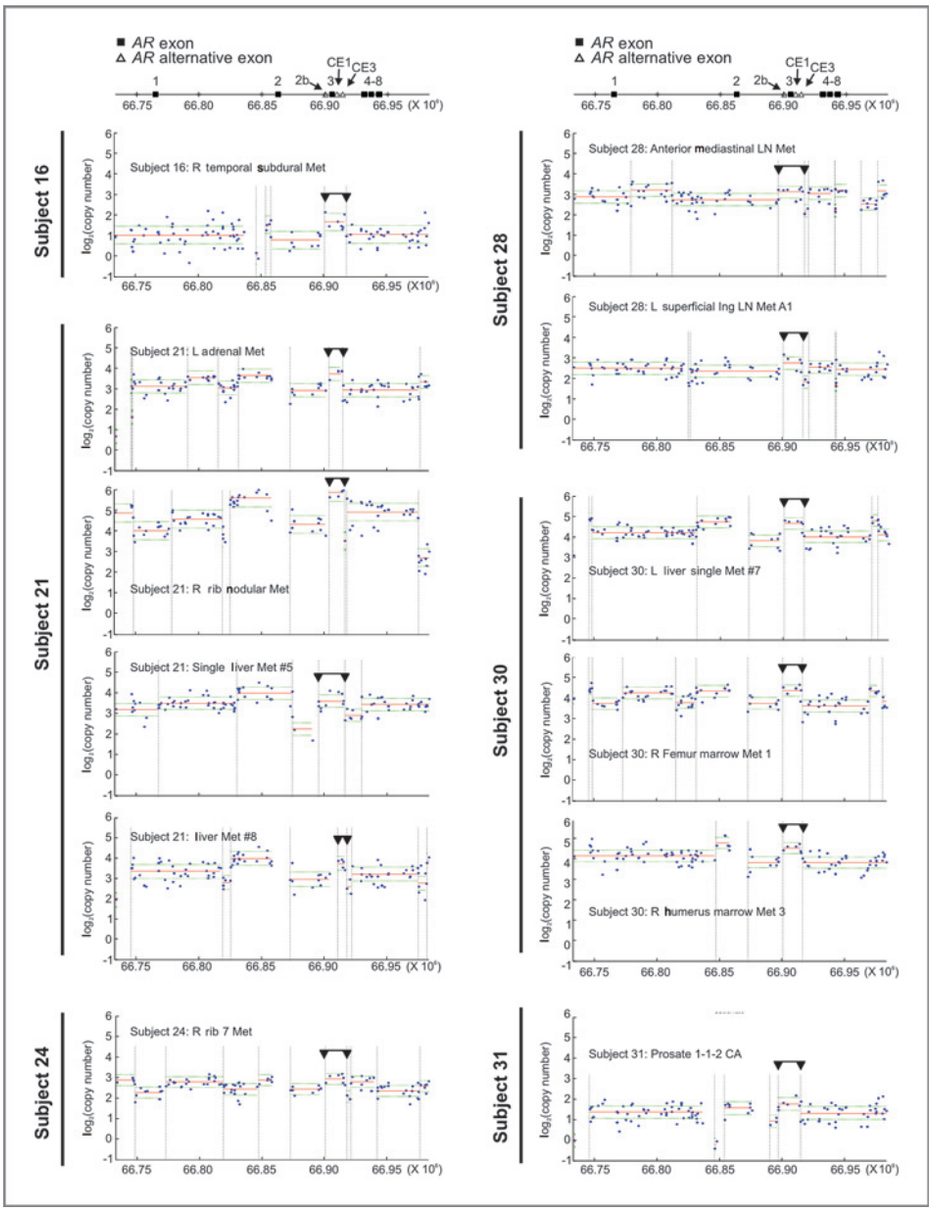

Figure 3. AR intragenic rearrangements in CRPCa detected by Affymetrix Genome Wide SNP 6.0 Array analysis of metastatic tissues. Top, exon organization of the *AR* locus on Xq11-12 and chromosome position (human genome build 19, hg19) is indicated at the top of each panel. All panels shown are individual tissue samples from CRPCa metastases. Blue dots, probe-level copy number; horizontal red lines, mean segment copy number; horizontal green dashed lines, SD; and dashed vertical lines, segment boundaries defined by the segmentation algorithm. Black horizontal lines with downward-facing arrowheads, a region of focal copy number alteration similar to 22Rv1 cells.

cells, we carried out higher-resolution copy number interrogation (Fig. 4A). Using this approach, we mapped the 5′ breakpoint between *AR* exons 2 and 2b to a resolution of 4 kb (Fig. 4B). Concurrently, we mapped the 3′ breakpoint between *AR* exons CE3 and 4 to a resolution of 8 kb (Fig. 4B). Attempts to map the 5′ or 3′ breakpoints with higher resolution yielded real-time PCR products associated with very low C_t values in both reference and test DNA samples, indicating repetitive sequence. Indeed, analysis of public reference genome sequence revealed long interspersed nuclear elements (LINE-1) and low complexity (TA)*n* repeats and AT-rich sequence in both of these regions (Fig. 4C).

It is common for the endpoints of genomic deletions or insertions to map to repetitive elements such as LINE-1, although the underlying mechanisms are not fully established (29, 30). One possibility is that extensive homology between LINE-1 elements at breakpoint junctions could lead to deletion on one sister chromatid and duplication on the other via nonallelic homologous recombination (NAHR; ref. 31). Pairwise alignments between the 5′ LINE-1 fragments and the full-length 3′ LINE-1 element identified a longer than 1-kb stretch of 87% sequence identity with one particular 5′ LINE-1 fragment, implicating NAHR as the basis for this rearrangement (Supplementary Fig. S7). Therefore, we conducted long-range PCR by using 2 pairs of outward facing

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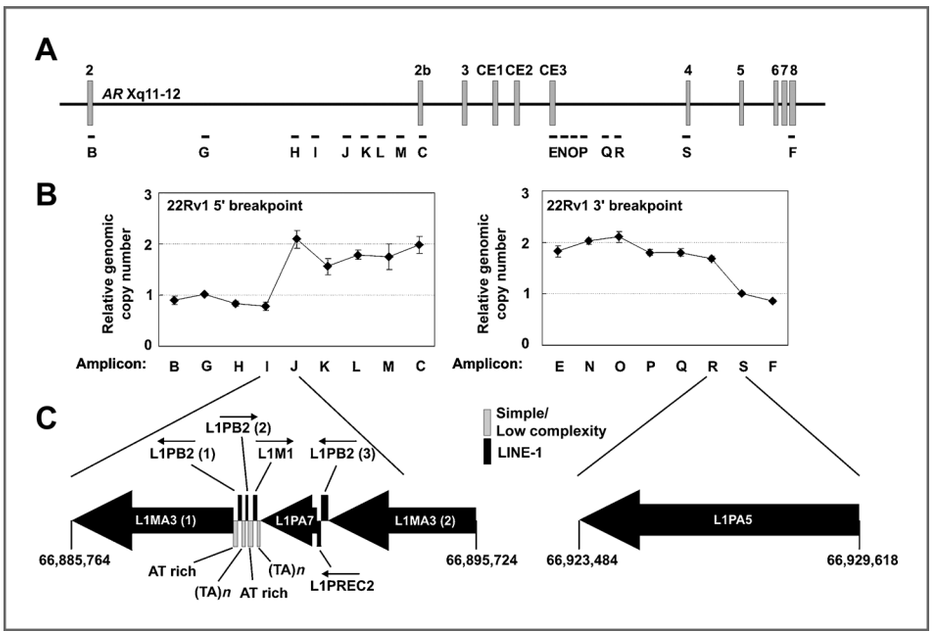

Figure 4. Fine mapping of *AR* intragenic rearrangement segment boundaries in 22Rv1 cells. A, schematic of the *AR* locus at Xq11-12. PCR amplicons used for copy number determination are labeled B, C, E, F, and G to S. B, genomic DNA from 22Rv1 cells was subjected to quantitative PCR, using amplicon primer pairs indicated in panel A. C_t values were converted to copy number by plotting sample C_t values on C_t versus copy number standard curves constructed from serial dilutions of BPH-1 genomic DNA. C, schematic of repetitive element organization at the 5′ and 3′ boundaries of the 22Rv1 duplicated AR segment in the reference human genome. Elements were defined by RepeatMasker 3.0 (41). Black arrows indicate the directional orientation of L1 elements, which are named on the basis of their evolutionary origin and sequence divergence, with relative ages (oldest to youngest) L1M1 > L1MA3 > L1PB2 > L1PREC2 > L1PA7 > L1PA5 (41, 42).

primers to isolate the breakpoint junction in 22Rv1 cells (Fig. 5A and Supplementary Fig. S8). This resulted in long PCR products of 6,723 and 4,762 bp (Supplementary Fig. S8). Sequencing of cloned PCR products revealed that they were identical over the common 4,762 bp and localized the 22Rv1-specific 5′ and 3′ breakpoints to genomic positions 66,889,976 and 66,924,525, respectively (Fig. 5B and C). Analysis of the break fusion junction revealed 27 bp of

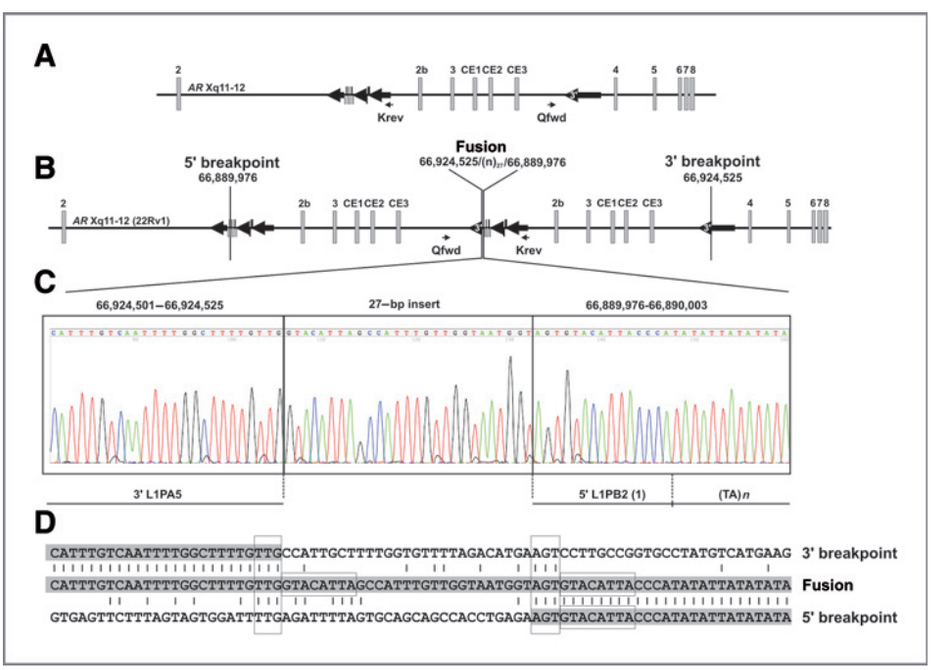

Figure 5. Outward-facing PCR to isolate the 22Rv1 *AR* tandem duplication. A, schematic of the *AR* locus at Xq11-12 with locations of primers used for outward-facing long-range PCR. B, schematic of the *AR* locus in 22Rv1 cells as revealed by sequencing of cloned long-range PCR products. C, electropherogram sequence of the *AR* break fusion junction in 22Rv1 cells, including a novel 27-bp insert. D, sequence alignments of the 3′ breakpoint, the 22Rv1 break fusion junction, and the 5′ breakpoint. Sequence contained in the break fusion junction is shaded in gray. Regions of microhomology are boxed.

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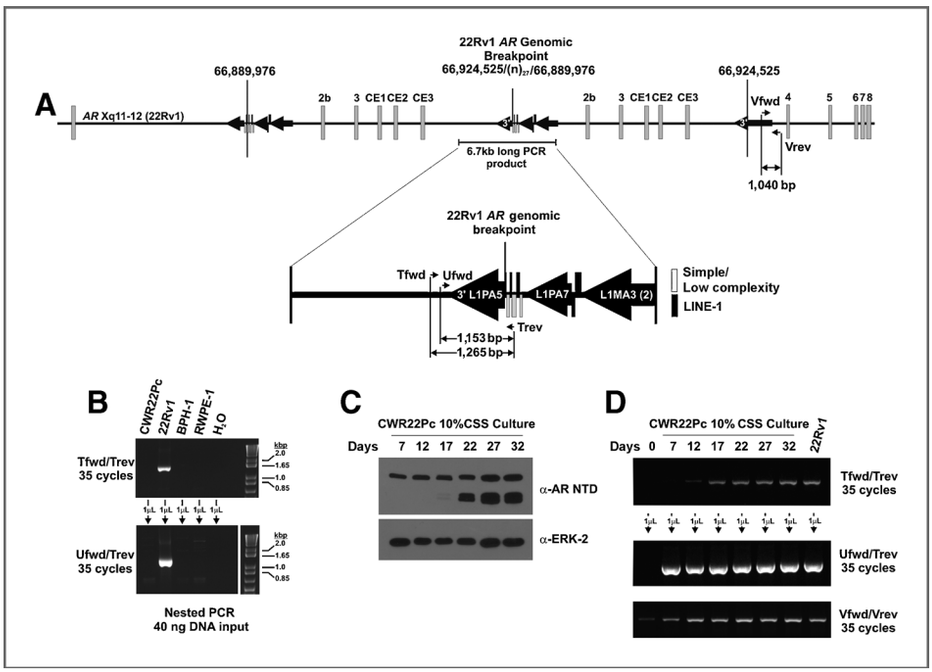

Figure 6. Concurrent emergence of *AR* intragenic rearrangement, androgen-independent growth, and high-level truncated AR isoform expression during CWR22Pc castration. A, schematic of the 22Rv1 *AR* locus and locations of primers used for nested PCR. B, conventional PCR was carried out using Tfwd/Trev primers and 40 ng of input DNA from the indicated cell lines. An aliquot of this reaction was used in a second nested PCR reaction using Ufwd/Trev primers. C, AR Western blot of CWR22Pc castration time course. CWR22Pc cells were cultured in androgen-depleted medium for the indicated time points. ERK-2, loading control. D, nested PCR of CWR22Pc castration time course. Reactions were carried out exactly as described in panel B.

inserted sequence (Fig. 5C). The origin of this sequence was not apparent by BLASTN and BLAT searches; however, the first 8 bp of this sequence perfectly matched an 8-bp motif at the 5′ breakpoint. Sequence alignments of the cloned break fusion junction and the 5′ and 3′ breakpoints showed virtually no extended homology through this region (Fig. 5D). However, regions of 3-bp microhomology were found at the breakpoints (Fig. 5D). Microhomology at the breakpoints and inserted sequence at the fusion site argues against NAHR and supports a microhomology-mediated break-induced replication (MMBIR; ref. 32) mechanism of segmental duplication in 22Rv1 cells.

Emergence of CRPCa cells during long-term CWR22Pc castration

Using conventional and nested PCR strategies, we confirmed that the *AR* breakpoint observed in 22Rv1 cells was indeed restricted to this cell line (Supplementary Fig. S8C and Fig. 6B). Previous studies have shown that androgen-dependent CWR22Pc xenograft tumors initially regress during castration but eventually recur with a CRPCa phenotype (23). To probe the link between *AR* intragenic rearrangement and CRPCa, we cultured CWR22Pc cells over a 1-month period in androgen-depleted medium. During the first 12 days of culture, no changes in AR protein expression patterns were observed (Fig. 6C). Interestingly, the 22Rv1 breakpoint was detected in CWR22Pc cells by nested PCR after 7 days of castration (Fig. 6D). The sensitivity of this nested PCR approach was determined to be as low as 1 to 2 genomes in limiting dilution assays (Supplementary Fig. S8D), indicating that the subpopulation of cells harboring this rearrangement was very rare. By day 17, discrete proliferative foci were apparent, which coincided with faint expression of truncated AR isoforms (Fig. 6C) and detection of the 22Rv1 breakpoint via conventional PCR (Fig. 6D). On day 17, cells were trypsinized and reseeded to disperse these proliferative foci. By day 22 and onward, androgen-independent cell growth was apparent, as was the expression of truncated AR isoforms. Together, these findings show that *AR* intragenic rearrangement is linked to high-level truncated AR isoform expression and CRPCa growth in a cell-based model of PCa progression.

Discussion

Recent reports describing the synthesis and function of truncated, constitutively active AR isoforms have provided a novel and conceptually simple mechanism for the resistance of CRPCa cells to androgen depletion (16–19). However, the mere presence of truncated AR isoforms does not correlate perfectly with androgen responsiveness, which highlights the importance of quantitative understanding in this area. This is especially apparent from a recent study showing that AR 1/2/3/CE3 [also termed AR-V7 (18) or AR-3 (17)] increases during progression to CRPCa but is also expressed in benign prostate tissue and hormone naive PCa (17). Because truncated AR isoforms were originally identified in CRPCa cells derived from the CWR22 model, the recent establishment of an androgen-dependent cell line from CWR22 xenografts has permitted an evaluation of the

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changes in *AR* mRNA splicing regulation that may occur during PCa progression in a lineage-related context. One striking difference between androgen-dependent CWR22Pc cells and 22Rv1 CRPCa cells was the expression profile of full-length and alternatively spliced *AR* mRNAs. Although alternatively spliced *AR* mRNAs and protein were detectable in both cell lines, we found that 22Rv1 cells had an enhanced capacity to efficiently synthesize *AR* 1/2/2b, *AR* 1/2/3/2b, and *AR* 1/2/3/CE3 mRNAs. We further showed tandem duplication of an approximately 35-kb segment harboring these alternative exons as a likely basis for the deregulation of AR mRNA splicing observed in 22Rv1 cells. Interestingly, a recent study identified 2 additional alternative exons expressed in VCaP cells that are clustered on this segment between AR exons CE1 and CE3 (20). Mechanistically, such a rearrangement could impair normal splicing by lengthening of the already vast distance between the *AR* transcription start site and *AR* exon 4, increasing the likelihood of incorporating 1 of the 2 sets of alternative exons preceding exon 4, disrupting the normal genomic organization of cis-acting intronic and exonic splicing elements or any combination of these possibilities. It will be important to elucidate a clear cause–effect mechanism, but technical limitations such as the size of the *AR* locus (~180 kb) and even larger aberrant locus in 22Rv1 cells (~215 kb) will have to be addressed.

AR overexpression is common in CRPCa, and *AR* gene amplification is thought to be a main driver of increased AR protein expression (33, 34). Most prior assessments of *AR* amplification in PCa tissues employed FISH, which lacks resolution and does not permit accurate copy number assessment along the length of the *AR* gene (7, 13, 34–36). Our findings indicate that a subset of amplified *AR* loci in CRPCa harbor intragenic rearrangements similar to 22Rv1, in addition to other alterations, which would clearly lead to a reconfigured *AR* exon organization for many of these alleles. It will therefore be important to conduct a comprehensive study of the relationship between *AR* intragenic rearrangements and levels of alternatively spliced AR isoforms in CRPCa to determine whether there is selection for intragenic rearrangement or whether intragenic rearrangement is simply arising as a by-product of *AR* gene amplification. Long-term castration of CWR22Pc cells suggests that *AR* intragenic rearrangement and the CRPCa growth phenotype are linked and that enrichment for cells with this genomic alteration occurs because of a selective advantage under castrate conditions. It is also possible that larger-scale genomic rearrangements may play a role in disrupted AR splicing, as evidenced by the recent identification of the truncated mAR-V4 isoform in the Myc-CaP mouse model, which results from alternative splicing of a cryptic exon nearly 1 Mb upstream of the mouse *AR* locus (20). Together, these findings indicate that tissues displaying *AR* intragenic rearrangements should be prioritized for further studies of AR splice variants and their importance to PCa prognosis and therapeutic response.

It will also be important to map and sequence the break fusion junctions within the *AR* locus in individual CRPCa metastases to obtain a more complete understanding of the mechanisms and significance of *AR* intragenic rearrangements. Our work revealed LINE-1 elements at the 5′ and 3′ ends of the 22Rv1 AR rearrangement. Transposable elements (TE) are implicated in the genesis of rearrangements underlying TE-related genetic diseases, including cancer (29), and often arise through NAHR. However, sequencing the 22Rv1 *AR* break fusion junction revealed a 27-bp insertion of unknown origin, which opposes a NAHR-based model. Indeed, stressed cancer cells are deficient in NAHR (37) and cancer-specific rearrangements frequently contain insertions ranging from 1 to 154 bp of so-called nontemplate sequence at the break fusion junction (38–40). Therefore, a new model, MMBIR has recently been proposed to account for this class of break fusion junctions in cancer cells (32).

In summary, our work describes a novel AR intragenic rearrangement in the 22Rv1 model of PCa progression, which is linked to enhanced synthesis of truncated AR isoforms and androgen-independent growth. We further show that similar genomic rearrangements occur in metastatic CRPCa specimens. It will be important in future studies to define whether intragenic *AR* rearrangements directly cause disrupted AR splicing, because a scenario of *AR* intralocus breaks leading to enhanced synthesis of truncated AR isoforms indicates that there may be little plasticity in the repertoire of AR isoforms synthesized. This would potentially limit the effectiveness of manipulating "alternative" splicing as a therapy for CRPCa. Nevertheless, a genomic basis for pathologic AR isoform expression may serve as a stable mechanism-based marker for resistance to androgen depletion therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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REVIEW

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Alternatively spliced androgen receptor variants

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Abstract

Alternative splicing is an important mechanism for increasing functional diversity from a limited set of genes. Deregulation of this process is common in diverse pathologic conditions. The androgen receptor (AR) is a steroid receptor transcription factor with functions critical for normal male development as well as the growth and survival of normal and cancerous prostate tissue. Studies of AR function in androgen insensitivity syndrome (AIS) and prostate cancer (PCa) have demonstrated loss-of-function AR alterations in AIS and gain-of-function AR alterations in PCa. Over the past two decades, AR gene alterations have been identified in various individuals with AIS, which disrupt normal AR splicing patterns and yield dysfunctional AR protein variants. Recently, altered AR splicing patterns have been identified as a mechanism of PCa progression and resistance to androgen depletion therapy. Several studies have described the synthesis of alternatively spliced transcripts encoding truncated AR isoforms that lack the ligand-binding domain, which is the ultimate target of androgen depletion. Many of these truncated AR isoforms function as constitutively active, ligand-independent transcription factors that can support androgen-independent expression of AR target genes, as well as the androgen-independent growth of PCa cells. In this review, we will summarize the various alternatively spliced AR variants that have been discovered, with a focus on their role and origin in the pathologic conditions of AIS and PCa.

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The androgen receptor: gene, mRNA, and protein

The androgen receptor (AR) is a 110 kDa member of the steroid receptor transcription factor family that mediates the cellular actions of the androgens testosterone and dihydrotestosterone (DHT). The structural organization of the AR gene, mature spliced mRNA, and protein domains are similar to the family members estrogen receptor α (ERα), ERβ, and progesterone receptor (Fig. 1; Lubahn *et al*. 1989). Males contain one copy of the AR gene located at chromosome position Xq11-12. AR exon 1 encodes the entire AR NH_2-terminal domain (NTD), which represents nearly 60% of the AR protein but is variable in length by virtue of polymorphic (CAG)*n* and (GGN)*n* repeat units encoding polyglutamine and polyglycine tracts respectively (Ferro *et al*. 2002, Ding *et al*. 2004, 2005). AR exon 2 encodes the first zinc finger in the AR DNA-binding domain (DBD), which is the DNA recognition helix that makes contact with major groove residues in an androgen-response element (ARE) half-site (Shaffer *et al*. 2004). AR exon 3 encodes the second zinc finger in the AR DBD, which is a dimerization interface that mediates binding with a neighboring AR molecule engaged with an adjacent ARE half-site (Shaffer *et al*. 2004). Exons 4–8 encode a short flexible hinge region and 11 α-helices folded in an α-helical sandwich to form the well-characterized AR COOH-terminal domain (CTD), which harbors the AR ligand-binding domain (LBD) and transcriptional activation function 2 (AF2) co-regulator binding interface (Matias *et al*. 2000, Sack *et al*. 2001, He *et al*. 2004, Hur *et al*. 2004, Estebanez-Perpina *et al*. 2005, Bain *et al*. 2006).

Each of these discrete protein domains plays an important functional role in the classical mode of AR activation by androgenic ligands. AR protein in the ligand-free state is localized in the cytoplasm, where it

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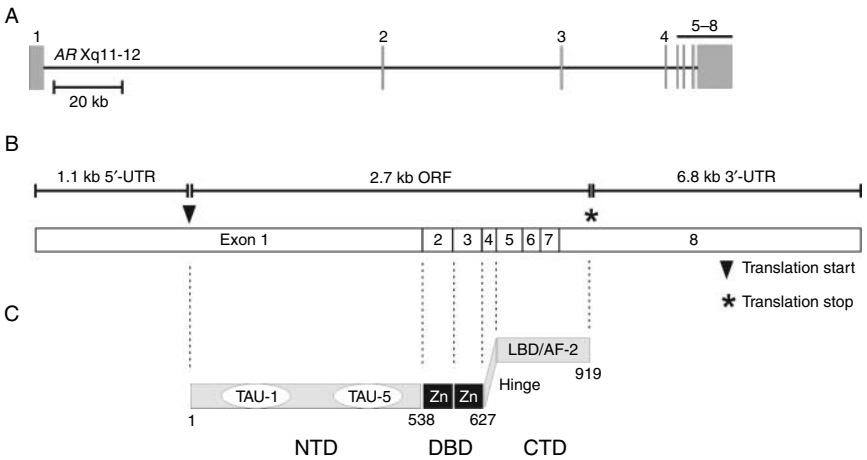

Figure 1 Modular organization of the AR gene, mRNA, and protein. (A) Exon organization of the ~180kb AR gene located on chromosome Xq11-12. (B) Normal splicing of AR exons 1-8 yields a 10.6kb mRNA species with large 5′ and 3′ untranslated regions (UTR) and a 2.7kb open reading frame (ORF). (C) The AR protein consists of a large AR NH_2-terminal domain (NTD) harboring transcriptional activation unit-1 (TAU-1) and TAU-5, a central DNA binding domain (DBD) consisting of two zinc fingers, and a COOH-terminal domain (CTD) harboring the AR ligand binding domain (LBD) and transcriptional activation function-2 (AF-2).

is engaged with Hsp90, other molecular chaperones, and high-molecular-weight immunophilins by virtue of interaction with the AR CTD (Pratt & Toft 1997, Smith & Toft 2008). Androgen binding induces a change in the conformation and composition of this multi-protein complex, which exposes the bipartite nuclear localization signal (NLS) in the hinge region (Zhou *et al.* 1994), thus allowing direct interaction with importin-α and subsequent nuclear translocation through the nuclear pore complex (Cutress *et al.* 2008). In the nucleus, AR binds as a dimer to AREs in promoter and enhancer elements of target genes, the best-characterized of which are prostate-specific antigen (PSA), TMPRSS2, and hK2 (Dehm & Tindall 2006). Transcriptional activation of these target genes is a complex, multi-step process that requires ordered-stepwise recruitment of a plethora of co-regulatory proteins (Heinlein & Chang 2004, Heemers & Tindall 2007). Although the AR AF2 domain is able to recruit well-characterized co-activators such as SRC-1, SRC-2/TIF-2, and SRC-3/AIB1, its contribution to transcriptional activity is relatively weak (He *et al.* 2004). In contrast, the AR NTD is a potent transcriptional activator, and this activity has been mapped to two primary transactivation domains termed transactivation unit 1 (TAU1) and TAU5. These two domains have been shown to be necessary and sufficient for full AR transcriptional activity (Callewaert *et al.* 2006). TAU1 activity has been more precisely mapped to a discrete five amino acid Leu-Lys-Asp-Ile-Leu (LKDIL) motif; however, the co-regulatory proteins that interact with this region have not been elucidated (Chamberlain *et al.* 1996, Callewaert *et al.* 2006). Our own work has mapped TAU5 activity to a discrete helical Trp-His-Thr-Leu-Phe (WHTLF) motif, and we have demonstrated that this region is important for AR transcriptional activity under conditions of no/low androgens (Dehm *et al.* 2007). Overall, the multi-protein complexes nucleated by active AR results in recruitment of the basal transcriptional machinery and a tightly controlled level of target gene transcription.

Naturally occurring AR variants: AR alternative splicing in normal tissues

Given the modular structure of the AR protein, and the important functional roles for discrete AR protein domains, it is anticipated that alternative splicing events would have profound effects on activity of the AR signaling axis. AR45 is a naturally occurring variant of the human AR, which was originally identified via 5′ rapid amplification of cDNA ends (RACE) with RNA isolated from human placenta tissue (Ahrens-Fath *et al.* 2005). AR45 mRNA was found to arise from alternative splicing of a previously unreported exon located within intron 1 of the AR gene (Fig. 2). This alternative exon, termed exon 1B, is situated ~22.1 kb downstream of AR exon 1 and can be spliced in place of AR exon 1 to yield a 45 kDa receptor isoform containing the entire AR DBD, CTD, and a novel seven amino acid

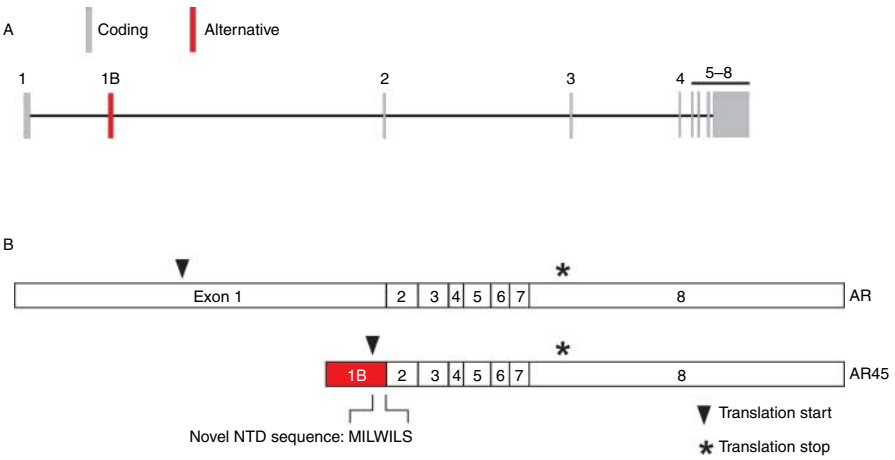

Figure 2 Alternative splicing of AR exon 1B gives rise to the AR45 mRNA isoform. (A) Schematic of the AR gene locus. The location of exon 1B is illustrated in red. (B) The AR45 mRNA is predicted to encode a NH_2-terminally truncated protein with an intact DNA binding domain and COOH-terminal domain.

Met-Ile-Leu-Trp-Leu-His-Ser sequence at the N-terminus in place of the wild-type AR NTD. RT-PCR analysis indicated that AR45 mRNA is expressed in diverse tissues including heart, muscle, uterus, prostate, lung, and breast, with no apparent expression in brain. However, these RT-PCR experiments were not quantitative, so it is unclear how AR45 expression levels compared with wild-type AR in these tissues. It is also unclear whether AR45 mRNA is translated into a protein in these tissues, although western blot analysis of LNCaP lysates demonstrated the presence of a ~45 kDa species that was immunoreactive with an antibody specific for the AR CTD. Functionally, ectopically expressed AR45 protein was shown to bind androgen, localize to the nucleus, interact with the full-length AR NTD, and inhibit full-length AR activity in a ligand- and DBD-dependent manner. Moreover, overexpression of AR45 in LNCaP cells inhibited proliferation. Together, these data indicate that AR45 is a negative regulator of AR signaling. However, AR45 was also demonstrated to harbor transcriptional activity under conditions of overexpression of the AR co-activators β-catenin or TIF-2 (Ahrens-Fath *et al*. 2005), which is a phenomenon that has been demonstrated for DBD/CTD fragments derived from the AR in other studies (Alen *et al*. 1999, He *et al*. 1999). A recent study revealed a potentially interesting role for AR45 in the increased risk of drug-induced cardiac arrhythmias in women. The human ether-a-go-go-related gene (HERG) K^+ channel has been implicated in this gender bias, and DHT-mediated stabilization of HERG protein was observed in cells transfected with AR45 but not full-length AR (Wu *et al*. 2008).

Loss-of-function AR variants: AR alternative splicing in androgen insensitivity syndrome

The X-linked inheritance pattern of androgen insensitivity syndromes (AIS) is caused by AR genetic alterations in 46, XY males. These AR gene alterations result in AR proteins with impaired function that prevents normal androgen signaling and proper development of internal and external male phenotypes (Brinkmann 2001). The most severe AIS phenotype is complete AIS (CAIS), where individuals have a complete female appearance. Partial AIS (PAIS) includes a wide range of phenotypes including individuals with a primarily female appearance as well as individuals with a primarily male appearance. In general, the severity of AIS is proportional to the level of impaired AR activity caused by specific alterations in the gene. The best-characterized AR gene alterations in AIS are single point mutations that result in an amino acid substitution or a premature translation termination, insertions/deletions resulting in a reading frameshift, or a complete/partial gene deletion (Gottlieb *et al*. 2004). However, a number of alternatively spliced AR mRNAs have been identified in AIS syndrome, resulting in AR proteins with impaired function. Importantly, gene defects have been associated with these alternatively spliced variants, often within intronic splice donor or splice acceptor sites (Fig. 3). Therefore, it would be more appropriate to refer to these events as disruptions in normal splicing as opposed to bona fide alternative splicing to reflect the fact that the pathologic transcripts are produced from an altered gene sequence.

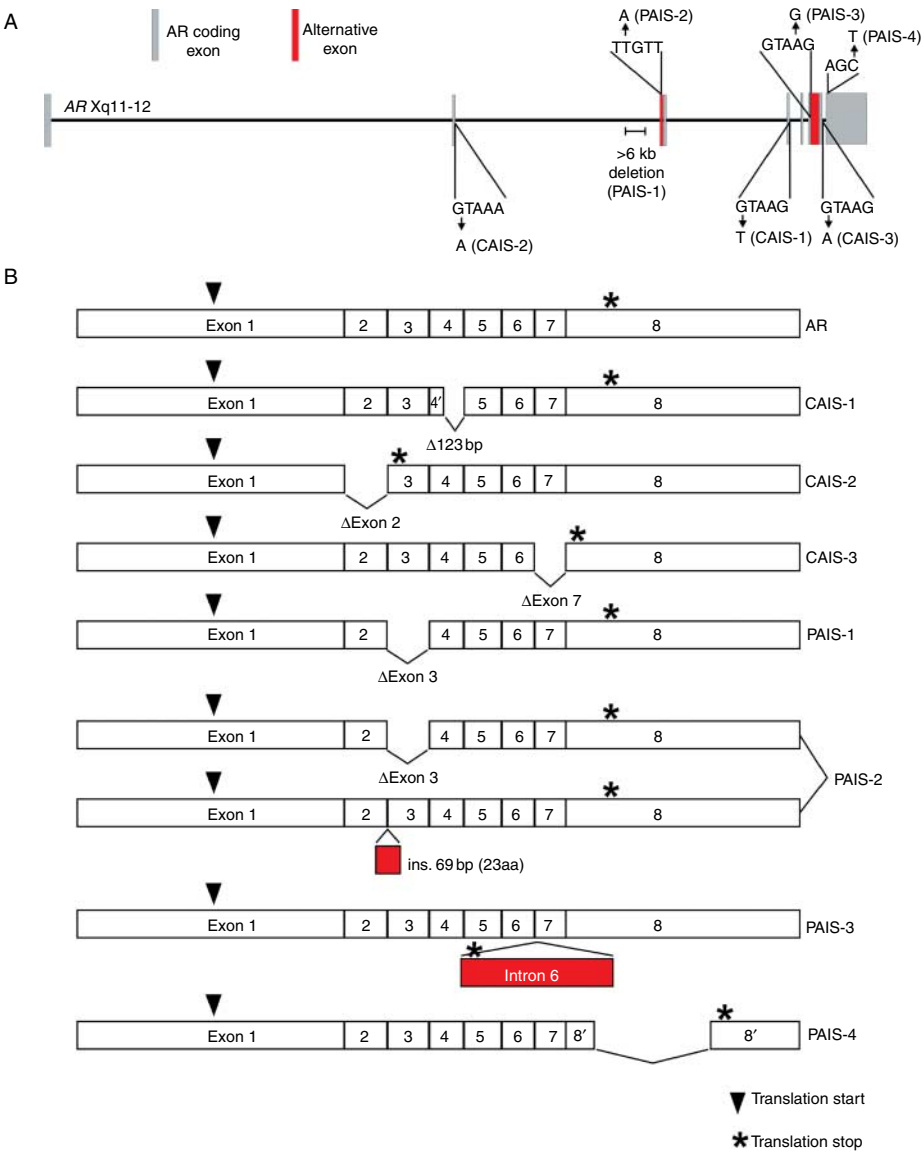

Figure 3 Mutations and structural alterations in the AR gene disrupt mRNA splicing patterns in androgen insensitivity syndrome (AIS). (A) Schematic of the AR gene locus with intronic point mutations and structural alterations that have been identified in individuals with complete AIS (CAIS) or partial AIS (PAIS). Predominant AR mRNA isoforms expressed in individuals with these specific gene alterations are illustrated in (B).

AR splicing disruptions in CAIS

In 1990, AR gene sequencing in a patient with CAIS yielded the observation that the sequences of coding exons were unaltered, but a G→T mutation occurred within the splice donor site of intron 4 (Ris-Stalpers *et al*. 1990). This mutation was shown to lead to the use of an alternative cryptic splice donor in exon 4, resulting in an in-frame 123 bp deletion from AR mRNA and a concomitant 41 amino acids internal deletion of the AR protein (CAIS-1 in Fig. 3). This variant displayed no ligand-binding activity and no transcriptional activity on an androgen-responsive promoter–reporter gene. Since this initial observation, similar mutations in splice donor sites of introns 2 and 7 have been described in two other individuals with CAIS, leading to skipping of exons 2 and 7 from AR mRNAs respectively (Lim *et al*. 1997, Hellwinkel *et al*. 1999). The exon 2-deleted AR protein (CAIS-2 in Fig. 3) was prematurely truncated by virtue of a premature stop codon encoded by out of frame exon 3, and the exon 7-deleted AR protein (CAIS-3 in Fig. 3) was ∼98 kDa as opposed to the 110 kDa wild-type

AR. As expected, these alternatively spliced AR variants were unable to bind androgens (Lim *et al*. 1997, Hellwinkel *et al*. 1999).

AR splicing disruptions in PAIS

Alternatively spliced AR variants have also been described in individuals with PAIS, indicating that cells with altered AR splicing patterns can retain some level of residual AR activity. This could result from AR variants maintaining some degree of transcriptional activity, or a residual level of splicing that permits synthesis of wild-type AR mRNA and protein. A >6 kb deletion has been described in AR intron 2, which resulted in skipping of exon 3 and synthesis of an AR variant protein (PAIS-1 in Fig. 3) that lacks the 39 amino acids zinc finger encoded by this exon (Ris-Stalpers *et al*. 1994). This variant displayed normal ligand binding, but no transcriptional activity on an AR-responsive promoter–reporter construct. PAIS was attributed to some residual splicing of AR exons 1–8 and a low level of expression of wild-type AR protein. Similarly, a T→A mutation 11 bp upstream of exon 3 was shown to be associated with two alternatively spliced AR transcripts (PAIS-2 in Fig. 3), one of which arose through skipping of exon 3, and one of which resulted from use of a cryptic splice acceptor site 71 bp upstream of exon 3 (Bruggenwirth *et al*. 1997). The protein encoded by use of this alternative splice acceptor site contained an additional 23 amino acids in-frame insert in the middle of the AR DBD directly between the two AR zinc fingers. This receptor was shown to bind androgens with normal affinity, but had no DNA-binding activity in a band shift assay. Interestingly, this same AR variant, named AR23, was detected in a metastatic prostate cancer (PCa) specimen via a yeast functional assay (Jagla *et al*. 2007). However, it is not clear whether this patient harbored a T→A mutation at position −11. In another individual, an A→G mutation in the splice donor site of intron 6 resulted in expression of larger AR mRNA species (PAIS-3 in Fig. 3) with retention of this intron (Sammarco *et al*. 2000). An in-frame stop codon 79 bp into this intron resulted in a smaller, truncated AR protein. As expected, androgen binding was impaired for this receptor variant, but no additional functional assays were performed. Finally, an additional PAIS splicing mutation that has been identified was a C→G mutation in the last position of codon 886 and concomitant replacement of the final 33 amino acids of the wild-type AR protein with novel eight amino acids (PAIS-4 in Fig. 3) as a result of a cryptic splice donor site (Hellwinkel *et al*. 2001). In this case, androgen-binding capacity was impaired and no androgen-induced transcriptional activity was observed on an androgen-responsive promoter–reporter gene.

Role of the AR in PCa development and progression

The prostate is an androgen- and AR-dependent tissue. PCa cells retain this androgen- and AR-dependent property. Androgen depletion therapy (ADT) is a collective term used to refer to surgical castration, pharmacologic castration via LH releasing hormone analogs, or administration of antiandrogens such as bicalutamide. These modes of ADT are the standard systemic treatments for locally advanced, relapsed, or metastatic PCa. Initially, these therapies are able to effectively inhibit AR transcriptional activity in PCa cells, leading to reduced expression of AR target genes such as PSA and tumor regression. However, changes eventually occur in the biology of tumors during ADT that allows resumption of tumor growth, and metastatic spread. This stage of the disease, termed castration-resistant PCa (CRPCa), is a major cause of PCa morbidity and mortality. Therefore, this transition from a treatable, androgen-dependent disease to a lethal, castration-resistant disease has been an area of intense interest. Studies on the mechanisms of PCa progression during ADT have demonstrated that in nearly all cases resumed AR transcriptional activity is a critical event (Knudsen & Scher 2009). Therefore, as appears to be the case with other targeted cancer therapies, resistance of PCa to ADT usually occurs through changes in the drug target. Several important mechanisms of AR reactivation during ADT have been identified including AR point mutations, intratumoral androgen production, and AR protein expression/gene amplification. These mechanisms of AR reactivation have been reviewed in detail elsewhere (Agoulnik & Weigel 2006, Culig & Bartsch 2006, Pienta & Bradley 2006, Heemers & Tindall 2007, Chen *et al*. 2009, Knudsen & Scher 2009). Investigation of intratumoral androgen production mechanisms and modes of AR activation have led to the development of abiraterone acetate and MDV3100 as new therapies for men with CRPCa. Abiraterone is an inhibitor of the CYP17 enzyme, which catalyzes two steps in androgen biosynthesis. This drug blocks intra-tumor conversion of progesterone and adrenal androgens to DHT (Attard *et al*. 2009) and a recent phase III trial demonstrated an overall survival benefit for abiraterone in men with metastatic CRPCa (de Bono *et al*. 2011). MDV3100 is a non-steroidal antiandrogen that can inhibit AR even under conditions of protein overexpression

(Tran *et al*. 2009). Phase I/II clinical trials have demonstrated efficacy for MDV3100 in a subset of patients with CRPCa, and this drug is currently being tested in phase III trials (Payton 2010, Scher *et al*. 2010). Overall, these clinical advances have served as confirmation that targeting AR signaling in CRPCa remains an important option for treatment of this stage of the disease.

Gain-of-function AR variants discovered in the CWR22 PCa model

It was recently discovered that AR alternative splicing can give rise to COOH-terminally truncated AR protein isoforms that lack the AR LBD. These AR isoforms are missing the region of the receptor that would be predicted to mediate responses to traditional ADT, as well as new AR-targeted therapies such as abiraterone and MDV3100. Key observations leading to this discovery were initially reported in 2002, when it was demonstrated that 22Rv1 cells expressed two separate AR protein species of ~112 and 75–80 kDa (Tepper *et al*. 2002). By antibody mapping, it was demonstrated that the smaller 75–80 kDa AR species was composed of the AR NTD and DBD, but lacked the AR LBD. Further biochemical characterization revealed that the AR ΔLBD isoform was constitutively nuclear and could bind DNA independent of androgens. Co-immunoprecipitation experiments indicated that the AR ΔLBD isoform did not interact with full-length AR, suggesting that this species functioned as an independent factor. In another study, western blots with benign and cancerous prostate tissue demonstrated that AR immunoreactive species with a similar mobility to the AR ΔLBD isoform were frequently expressed in clinical PCa (Libertini *et al*. 2007). Together, these studies provided the first evidence that AR protein species lacking the LBD could play an important role in modulating PCa therapy resistance.

The mechanism proposed for synthesis of the AR ΔLBD protein species in PCa cells was via proteolytic degradation of full-length AR by calpain-2 (Libertini *et al*. 2007). This was based on the presence of a consensus calpain cleavage site in the AR hinge region and the observation that calpain-2 was able to cleave full-length AR protein in cell extracts derived from LNCaP and 22Rv1 cells, as well as the CRPCa CWR-R1 cell line which was also derived from the CWR22 xenograft model (Libertini *et al*. 2007, Chen *et al*. 2010). In addition, the full-length AR mRNA in 22Rv1 cells had been shown to be larger due to tandem duplication of AR exon 3, and this larger form of the AR appeared to have enhanced susceptibility to this mechanism of cleavage (Libertini *et al*. 2007). However, several recent studies have suggested that alternative splicing could also be an important contributor to the synthesis of truncated AR species lacking the AR LBD in these and other cells. This new concept was initially based on the observation of differential siRNA targeting of the various AR species in 22Rv1 cells (Dehm *et al*. 2008). For example, siRNA targeted to AR exon 7 abolished expression of full-length AR in 22Rv1 cells, but had no effect on the smaller 75–80 kDa species. Conversely, siRNA targeted to AR exon 1 knocked down expression of all AR immunoreactive species in 22Rv1 cells. Functionally, androgen-dependent expression of AR target genes and androgen-dependent cell growth was shown to be attributable to full-length AR, whereas androgen-independent expression of AR target genes and constitutive, androgen-independent growth was shown to be supported by the smaller 75–80 kDa species. These observations strongly suggested that different mRNA species encoded the different AR protein species observed in these cells, and provided the foundation for 3′-RACE and other approaches to identify their origin (Dehm *et al*. 2008, Guo *et al*. 2009, Hu *et al*. 2009, 2011, Marcias *et al*. 2010). These efforts led to the identification of a series of alternatively spliced AR mRNAs expressed in 22Rv1 cells that resulted from cryptic exons located within AR introns 2 and 3. The AR isoforms or variants encoded by these alternatively spliced mRNAs have been shown to function as constitutively active, ligand-independent transcription factors that can support the CRPCa phenotype in various model systems. Specific details of the individual AR isoforms that have been identified in models derived from the CWR22 xenograft are discussed in the following section. A major barrier to clarity regarding this emerging topic is the nomenclature that has been employed by different investigators, leading to instances where different names have been given to the same isoform (e.g. AR 1/2/3/2b, AR-V4, AR5, and ARV6 are all encoded by contiguously spliced AR exons 1/2/3/2b; AR-V7 and AR3 are both encoded by contiguously spliced AR exons 1/2/3/CE3; and AR^{v567es} and AR-V12 are both encoded by skipping of exons 5–7). Also, there are instances where different isoforms have been given the same name (e.g. AR-V7 encoded by contiguously spliced AR exons 1/2/3/CE3 and AR-V7 encoded by contiguously spliced AR exons 1/2/3/3′/4/5/6/7/8). Therefore, we will discuss these isoforms in the context of their exon constituency, and acknowledge all of the various names that have been given to these isoforms to date (Fig. 4).

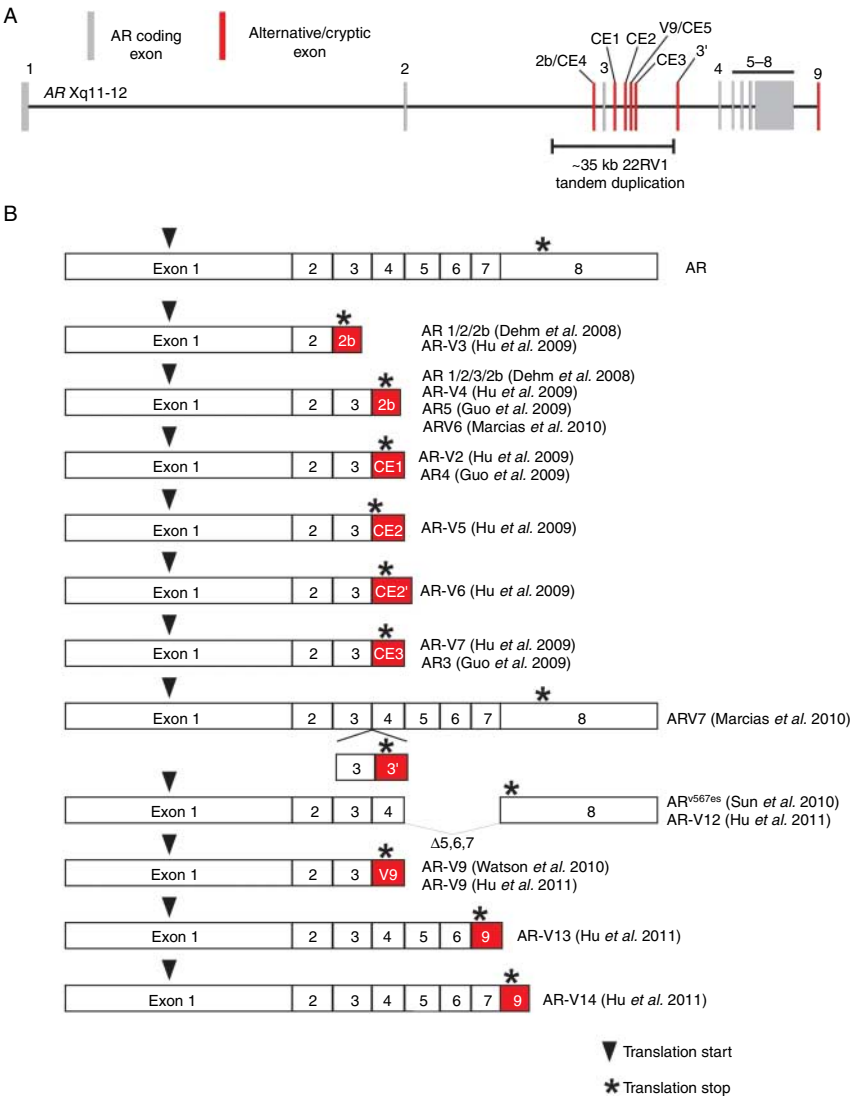

Figure 4 Alternatively spliced AR isoforms identified in prostate cancer (PCa). (A) Schematic of the AR gene locus with locations of alternatively spliced, cryptic exons illustrated in red. The location of an intragenic tandem duplication identified in 22Rv1 cells is indicated. (B) Alternative splicing of cryptic exons in the AR locus or exon skipping gives rise to COOH-terminally truncated AR mRNA isoforms that encode constitutively active, ligand-independent transcription factors. CE2′ denotes the use of an alternative splice acceptor site for splicing of exon CE2 downstream from exon 3. Additional alternatively-spliced mRNA isoforms which have not been cloned or characterized are not illustrated in this figure, but are discussed in the text.

Splicing of AR exons 1/2/2b and 1/2/3/2b

AR exon 2b was discovered following 5′-RACE experiments with RNA derived from 22Rv1 cells (Dehm *et al*. 2008). AR exon 2b was shown to splice downstream of either AR exon 2 or AR exon 3, giving rise to truncated AR proteins containing the AR NTD, one or both zinc fingers of the AR DBD, and a short 11 amino acid COOH-terminal extension encoded by AR exon 2b. Expression of the 75–80 kDa immunoreactive species in 22Rv1 cells was reduced upon transfection with exon 2b-specific siRNA, which corresponded with an inhibition of androgen-independent cell proliferation. These data confirmed that transcripts containing AR exon 2b were translated to functional proteins. Importantly, there was no effect of exon 2b siRNA on androgen-stimulated proliferation, further supporting the concept that truncated AR isoforms could support features of the CRPCa phenotype independent of full-length AR. Functionally, AR 1/2/2b and AR 1/2/3/2b were shown to

function as constitutively active transcription factors in promoter–reporter assays. The AR 1/2/2b AR mRNA was also shown to be expressed in VCaP cells as well as the LuCaP 23.1 and 35 models of PCa progression.

Additional studies by other groups have confirmed the existence of mRNA species containing exon 2b in the 22Rv1 model system, but with variability in the precise 3′ composition of the transcript. For example, one transcript was reported with exon 2b (referred to as exon CE4 in the study) spliced between exons 2 and 3, with exon CE1 at the 3′ terminus (Hu *et al*. 2009). This isoform was termed AR-V3 in this study, and was proposed to encode a 53 amino acids COOH-terminal extension due to a TAA trinucleotide deletion in the sequence of CE4 compared with the sequence of exon 2b. The reason for this discrepancy is not clear, but could be due to procedural differences or the fact that 22Rv1 cells harbor 2 genomic copies of AR exon 2b (Li *et al*. 2011). Another transcript was also identified with exon 2b spliced between the duplicated copies of exon 3, with exon CE1 at the 3′ terminus (Hu *et al*. 2009). This configuration, termed AR-V4, matched the splicing pattern for the AR5 mRNA reported in a separate study (Guo *et al*. 2009). Importantly, AR5 mRNA expression was detected by RT-PCR at a low level in benign prostate tissue as well as PCa tissue. In yet another study, AR exon 2b (referred to as exon 2′ in that study) was shown to be spliced between the duplicated copies of exon 3 in the context of an mRNA species, termed V6, with contiguous splicing of AR exons 1–8 (Marcias *et al*. 2010). Together, these data indicate that many AR mRNAs in the 22Rv1 cell line contain exon 2b spliced after exon 2 or 3, with possible variation in the composition of the 3′ untranslated region of these transcripts.

Splicing of AR exons 1/2/3/CE1 and 1/2/3/3/CE1

Two AR mRNA species composed of contiguously spliced AR exons 1/2/3/CE1 (termed AR-V1) and AR exons 1/2/3/3/CE1 (termed AR-V2) were demonstrated to be expressed in 22Rv1 cells following the bioinformatic identification of AR exon CE1 as an expressed sequence tag mapping to AR intron 3 (Hu *et al*. 2009). The AR-V1 transcript composed of AR exons 1/2/3/CE1 was detected by quantitative RT-PCR at a higher level in RNA isolated from CRPCa tissue specimens versus hormone naïve PCa. In another study, the AR 1/2/3/CE1 isoform was also detected in 22Rv1 cells, and termed AR4 (Guo *et al*. 2009). No studies have been performed to demonstrate translation of either of these mRNAs into endogenous proteins.

Splicing of AR exons 1/2/3/CE2

Two separate AR mRNA species composed of contiguously spliced AR exons 1/2/3/CE2 have been identified, with different locations for the splice acceptor site used for incorporation of exon CE2 into these mRNAs (Hu *et al*. 2009). As with the identification of AR exon CE1, exon CE2 was originally discovered by bioinformatic identification of an expressed sequence tag mapping to AR intron 3. The isoform generated from utilization of the most 5′ splice acceptor site in exon CE2, termed AR-V5, results in a protein predicted to encode a single aspartate residue following exon 3 coding sequence. The isoform generated from utilization of the most 3′ splice acceptor site in exon CE2, termed AR-V6, results in a protein predicted to encode a six amino acid COOH-terminal extension following exon 3 coding sequence. No studies have been performed to demonstrate translation of this mRNAs into an endogenous protein.

Splicing of AR exons 1/2/3/CE3

The best-characterized alternatively spliced AR isoform identified to date is encoded by contiguously splicing of AR exons 1/2/3/CE3. In one study, this isoform was named AR-V7 (Hu *et al*. 2009), and in another study this same isoform was named AR3 (Guo *et al*. 2009). As with AR exons CE1 and CE2, exon CE3 was originally discovered based on bioinformatic identification of an expressed sequence tag mapping to AR intron 3 (Hu *et al*. 2009). Importantly, AR 1/2/3/CE3 mRNA was detected in various clinical PCa specimens as well as normal prostate tissue (Hu *et al*. 2009). Quantitative RT-PCR demonstrated that the RNA levels of this isoform could predict biochemical recurrence following surgery (Hu *et al*. 2009). The 16 amino acids COOH-terminal extension encoded by AR exon CE3 has been amenable to antibody development (Guo *et al*. 2009, Hu *et al*. 2009), and the utilization of these isoform-specific antibodies has revealed widespread protein expression of this isoform in cell lines, xenografts, and clinical specimens. Importantly, protein expression of this isoform was shown to be increased in CRPCa versus hormone naïve PCa (Guo *et al*. 2009). Also, cytoplasmic levels of this isoform could predict biochemical recurrence following surgery (Guo *et al*. 2009). It is interesting to note that the AR 1/2/3/CE3 isoform is also expressed at the mRNA and protein level in benign prostate tissue, indicating that there may also be a role for this splicing event in normal cells (Guo *et al*. 2009). Functionally, AR 1/2/3/CE3 was demonstrated to function as a

constitutively active, ligand-independent transcription factor that could induce a CRPCa growth phenotype in LNCaP cells grown *in vitro* and as xenografts *in vivo* (Guo *et al*. 2009). Interestingly, in one study, gene expression profiling of LNCaP cells transfected with an expression vector encoding AR 1/2/3/CE3 demonstrated that the ligand-independent transcriptional activity of this isoform could largely recapitulate the gene expression program activated by ligand-induced full-length AR, including the PSA gene (Hu *et al*. 2009). However, another study that used shRNAs to selectively knockdown the AR 1/2/3/CE3 or full-length AR isoforms in 22Rv1 and CWR-R1 cells demonstrated that AR 1/2/3/CE3 activated a significantly different gene expression program than ligand-activated AR (Guo *et al*. 2009). One differentially regulated gene that was identified was AKT1 (Guo *et al*. 2009). The AR 1/2/3/CE3 isoform was shown to activate AKT1 expression at the mRNA and protein level, whereas full-length AR had no effect on AKT1 expression. Mechanistically, this could be attributed to the finding that AR 1/2/3/CE3 but not full-length AR could bind to two AREs located in the AKT1 promoter region. Overall, this body of work demonstrates that an AR variant derived from contiguous splicing of AR exons 1/2/3/CE3, termed AR-V7 or AR3, is a clinically validated AR isoform that could play an important role in supporting the CRPCa phenotype.

Insertion of an extra copy of exon 3 and a novel exon 3′ between AR exons 3 and 4

Additional alternatively spliced AR isoforms have recently been cloned from 22Rv1 cells using a yeast functional assay to identify AR cDNAs with constitutive or ligand-induced transcriptional activity (Marcias *et al*. 2010). This approach led to the identification of an additional cryptic exon in AR intron 3 termed 3′. Sequencing of the isolated cDNA, which was named AR-V7 in this study, demonstrated that exon 3′ spliced between an extra copy of AR exons 3 and 4, giving rise to a truncated AR isoform containing the entire AR NTD, DBD, an extra zinc finger encoded by the extra copy of exon 3, and a novel 23 amino acids COOH-terminal extension encoded by exon 3′.

Gain-of-function AR variants discovered in other PCa models

The identification of alternatively spliced, truncated AR isoforms lacking the AR LBD in cell lines derived from the CWR22 PCa xenograft model, and extension of the findings to other models of PCa progression as well as human tissues demonstrated that this phenomenon may be a widespread mechanism of resistance to ADT therapies targeting the AR LBD. Indeed, examination of AR mRNA expression profiles in various other PCa models has led to the identification of additional AR mRNA species that are predicted to encode proteins that display constitutive, ligand-independent transcriptional activity. These isoforms and key details of their discovery are outlined below.

Splicing of AR exons 1/2/3/4/8 in LuCaP 86.2 and LuCaP 136 xenografts

An AR mRNA isoform arising through skipping of exons 5–7, termed AR^{v567es}, was discovered in the LuCap 86.2 and LuCaP 136 xenografts following examination of AR mRNA profiles via RT-PCR (Sun *et al*. 2010). Interestingly, in the LuCaP 86.2 xenograft, full-length AR mRNA did not appear to be expressed at either the mRNA or protein level, which is in contrast to 22Rv1 and other models where full-length AR expression levels remain high. No experiments have been performed to demonstrate that the AR^{v567es} mRNA is translated to an endogenous protein in these xenograft models, although the xenografts do display high-level expression of a ∼80 kDa species that is immunoreactive with an AR NTD-directed antibody (Sun *et al*. 2010). Skipping of AR exons 5–7 was confirmed via RT-PCR in several additional xenograft models of PCa progression, patient samples of metastatic CRPCa, primary PCa, as well as benign prostate epithelium. These findings indicate that skipping of exons 5–7 by the splicing machinery is frequent, and can even occur in non-cancerous tissue. Functionally, AR^{v567es} was shown to act as a constitutively active, ligand-independent transcription factor that could support CRPCa cell growth *in vitro* and *in vivo*. Interestingly, AR^{v567es} was also shown to interact with full-length AR and enhance its ligand-dependent and ligand-independent activity, perhaps through a mechanism of protein stabilization or even induction of full-length AR translocation to the nucleus in the absence of androgens.

Deep sequencing identifies additional alternative AR splicing patterns in VCaP cells

The VCaP cell line has been an additional cell-based PCa model of interest for studying AR alternative splicing in PCa progression because these cells express a smaller 75–80 kDa AR species that is recognized by antibodies directed to the AR NTD, but not the LBD (Dehm *et al*. 2008). An adaptation of the 3′-RACE

method was recently described wherein 3′-RACE products produced using a forward primer spanning the AR exons 2/3 junction were directly sequenced using 454 and SOLiD next-generation sequencing platforms (Watson *et al*. 2010). This approach confirmed splicing of AR exons CE1 and CE3 downstream of exon 3 in VCaP cells. However, this approach also identified four additional isoforms, named AR-V8 through AR-V11, that resulted from exon 3 read-through, splicing of novel cryptic exons in AR intron 3, or use of a different splice acceptor site in exon CE3. Similar to previously identified AR variants, these novel mRNAs would be predicted to encode variable-length COOH-terminal extensions following exon 3 sequence. Additional RNA species were identified in VCaP cells that appeared to arise through exon skipping, and splicing of a novel cryptic exon within AR intron 5. No full-length cDNAs were isolated from VCaP cells that corresponded to these splicing events detected by deep sequencing, so it is unclear whether these putative new AR isoforms represent splicing intermediates, splicing errors targeted for nonsense-mediated RNA decay, or bona fide alternatively spliced AR mRNAs that encode functional proteins. However, this work demonstrated the power of using next-generation sequencing technology for studying AR splicing events in PCa samples.

Deep sequencing identifies alternative AR splicing patterns in Myc-CaP cells

The development of the 3′-RACE/next-generation sequencing workflow using VCaP cells led to deployment of this approach in the Myc-CaP model of PCa. Myc-CaP cells were derived from prostate tumors that arose in mice expressing a prostate-specific myc transgene (Watson *et al*. 2005). A recent study demonstrated the synthesis of four novel alternatively spliced AR isoforms in these cells, which were named mAR-V1-4 (Watson *et al*. 2010). mARV1 was composed of contiguously spliced mouse exons 1/2/3, followed by read-through into intron 3. Remarkably, mAR-V2 and mAR-V4 were found to harbor novel exons located outside of the AR gene. One exon, located ∼250 kb downstream of the AR locus, was shown to be spliced after AR exon 3 to yield the mAR-V2 isoform. The other exon, located ∼1 Mb upstream of the AR locus, was shown to be spliced after AR exon 4 to yield mAR-V3 and mAR-V4. mAR-V3 differed from mAR-V4 by virtue of skipping of AR exon 3. Importantly, expression of mAR-V2 and mAR-V4 was confirmed at the protein level following isoform-specific knockdown of these species in Myc-CaP cells. Functional study of the mAR-V2 and mAR-V4 isoforms demonstrated that mAR-V4 was a constitutively active, ligand-independent transcription factor, and was constitutively localized to the nucleus. Conversely, mAR-V2 did not display transcriptional activity and was found to be localized predominantly to the cytoplasm. Consistent with these findings, ectopic expression of mAR-V4, but not mAR-V2, could support the growth of LNCaP xenografts in castrated mice. Interestingly, studies with the AR antagonist MDV3100 and siRNA directed to the full-length AR mRNA demonstrated that the CRPCa-promoting effects of mAR-V4 required functional full-length AR. Similar observations were made with the AR 1/2/3/CE3 isoform, indicating that truncated AR variants may not be able to take over the complete function of full-length AR under castrate conditions.

Genomic tiling arrays identifies alternative AR splicing patterns in clinical CRPCa

Another novel workflow that has been developed to identify additional alternatively spliced AR isoforms is based on selective linear amplification of sense RNA using an exon 3-anchored forward primer, followed by detection of amplified products using an AR gene tiling array (Hu *et al*. 2011). This approach was able to detect synthesis of mRNAs containing AR exons 2b/CE4, CE1, CE2, and CE3 in 22Rv1 cells, but also detected expression of a novel isoform termed AR-V9, which was produced by contiguous splicing of AR exons 1/2/3 and a novel cryptic exon termed CE5, located within AR intron 3. This exon configuration matched the AR-V9 isoform previously reported in VCaP cells (Watson *et al*. 2010). This approach also led to the identification of an additional novel AR exon expressed in RNA derived from CRPCa tissue specimens. This exon, termed ‘exon 9’ was located downstream of AR exon 8, and was shown to be the most 3′ exon in three discrete AR mRNA species, termed AR-V12 (AR exons 1/2/3/4/8/9, which would encode the AR^{v567es} variant), AR-V13 (AR exons 1/2/3/4/5/6/9), and AR-V14 (AR exons 1/2/3/4/5/6/7/9; Hu *et al*. 2011). Functional evaluation demonstrated that AR-V9 and AR-V12 (identical to AR^{v567es}) displayed varying degrees of constitutive, ligand-independent transcriptional activity depending on the cell line studied, and AR-V9 was shown to function independently of full-length AR in LNCaP cells.

Regulation of AR splicing and synthesis of truncated AR isoforms

Truncated AR mRNA and/or protein expression, in particular the AR-V7/AR3 isoform resulting from splicing of AR exons 1/2/3/CE3 and the AR^{v567es} isoform, resulting from skipping of AR exons 5–7, has been shown to occur in normal prostate tissues, indicating that the mere presence of these factors is unlikely to be pathogenic. This observation also suggests that there may be normal functions attributable to AR alternative splicing and synthesis of truncated AR isoforms. This possibility is also supported by studies of the AR45 isoform, which is synthesized at the mRNA level in normal tissues of various origin. However, a consistent finding has been an increased level of expression of alternatively spliced, truncated AR isoforms in CRPCa cells versus androgen-dependent PCa cells (Dehm *et al*. 2008, Guo *et al*. 2009, Hu *et al*. 2009, Sun *et al*. 2010). This suggests that ADT exerts a selective pressure favoring the expression of constitutively active, truncated AR isoforms. One possible mechanism for this increase in truncated AR isoform expression could be changes in expression or activity of factors that regulate AR splicing patterns. In this case, plasticity in these splicing patterns would be expected, which represents an opportunity to develop therapies to modify aberrant splicing patterns. However, studies of alternatively spliced AR variants synthesized in individuals with AIS has demonstrated that alterations in AR splicing patterns can also be stable due to sequence or structural changes in the genomic DNA template, which represents a 'new normal' for that cell. With these concepts in mind, our laboratory recently reported that the disruptions in AR splicing patterns in 22Rv1 cells was linked to a ~35 kb intragenic tandem duplication of a genomic segment encompassing AR exon 3 and many of the cryptic AR exons that are expressed in these and other cells (Li *et al*. 2011). These include exons 2b/CE4, CE1, CE2, CE3, CE5, as well as the four additional exons that were recently identified in the AR-V8-V11 isoforms expressed in VCaP cells. This rearrangement was not observed in the CWR22Pc cell line, which is an androgen-dependent subline developed from the original CWR22 xenograft (Li *et al*. 2011). However, during long-term castration, CWR22Pc cells progressed to a CRPCa growth phenotype that was marked by emergence of a rare subpopulation of cells harboring this gene rearrangement, and emergence of high-level truncated AR expression (Li *et al*. 2011). This finding demonstrates that alterations in the AR gene may underlie disruptions in mRNA splicing patterns observed in CRPCa cells such as 22Rv1. This concept is also supported by the observation that the mAR-V4 isoform results from contiguous splicing of mouse AR exons 1/2/3/4, and a cryptic exon located ~1 Mb upstream of the AR locus, which, similar to the AR 1/2/3/2b isoform, likely requires alterations in gene architecture to be synthesized (Watson *et al*. 2010). Remarkably, the AR gene in Myc-CaP cells, where mAR-V4 was discovered, is frequently amplified, indicating that amplification may also be associated with alterations in gene architecture (Watson *et al*. 2005). By examining high-resolution gene copy number data derived from clinical CRPCa specimens, we observed frequent copy number imbalances along the length of amplified AR genes, which is a finding consistent with complex gene rearrangements (Li *et al*. 2011). Together, these data suggest that changes in the genomic DNA template may underlie disrupted AR splicing patterns and pathologic synthesis of truncated, constitutively active AR isoforms in CRPCa cells. Further studies are required for a more complete understanding of the mechanisms regulating synthesis of alternatively spliced, truncated AR isoforms in PCa.

Perspectives and future directions

Various alternatively spliced AR isoforms have been identified in pathologic conditions including AIS and CRPCa. Alternatively spliced AR isoforms have also been observed in non-cancerous tissues; however, it remains to be determined whether these have a role in normal cellular processes. In CRPCa, a common theme is the synthesis of AR isoforms with a modular organization consisting of the AR NTD, the AR DBD, and short COOH-terminal extensions of variable length and sequence. Constitutive, ligand-independent transcriptional activity of these isoforms represents a conceptually simple mechanism for the development of resistance to PCa therapies that target the LBD. However, several studies have clearly demonstrated that additional understanding is required in this area. For example, there is evidence to suggest that the exons encoding the COOH-terminal extensions of truncated AR isoforms may provide important regulatory information in addition to harboring in-frame translation stop codons. This is particularly important because replacement of AR exon 4 with an alternative cryptic exon would disrupt the well-characterized AR NLS encoded by correctly spliced exons 3 and 4. Future studies are required to resolve this issue, and determine the molecular basis for the differences in

nuclear localization of the different AR variants that have been reported (Sun *et al*. 2010, Watson *et al*. 2010). Similarly, differences in transcriptional activity of different AR variants have been reported (Dehm *et al*. 2008, Guo *et al*. 2009, Watson *et al*. 2010, Hu *et al*. 2011). It will also be important to understand whether these different COOH-terminal extensions adjacent to the AR DBD can influence DNA-binding activity. The mechanisms by which full-length AR could influence AR isoform function (and *vice versa*) will also require further investigation, particularly in light of the findings that the AR^{v567es} isoform interacts with wild-type AR (Sun *et al*. 2010) and that interfering with full-length AR expression or activity affects the function of the ectopically expressed AR-V7 and mAR-V4 isoforms (Watson *et al*. 2010). However, these findings contrast with studies in models that naturally express truncated AR isoforms. For example, the LuCaP 86.2 xenograft model does not appear to express full-length AR, indicating that normal AR activity is not required for AR^{v567es} function (Sun *et al*. 2010). Similarly, knockdown of full-length AR in 22Rv1 cells does not affect androgen-independent proliferation or constitutive activation of AR target genes supported by truncated AR isoforms (Dehm *et al*. 2008). Finally, it will be critical in future studies to evaluate quantitatively the levels of alternatively spliced AR isoforms relative to wild-type AR to differentiate between background splicing byproducts and significant alterations in AR splicing patterns that could affect response to PCa therapies targeting the AR LBD.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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ORIGINAL ARTICLE

AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression

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Reactivation of the androgen receptor (AR) during androgen depletion therapy (ADT) underlies castration-resistant prostate cancer (CRPCa). Alternative splicing of the *AR* gene and synthesis of constitutively active COOH-terminally truncated AR variants lacking the AR ligand-binding domain has emerged as an important mechanism of ADT resistance in CRPCa. In a previous study, we demonstrated that altered AR splicing in CRPCa 22Rv1 cells was linked to a 35-kb intragenic tandem duplication of AR exon 3 and flanking sequences. In this study, we demonstrate that complex patterns of *AR* gene copy number imbalances occur in PCa cell lines, xenografts and clinical specimens. To investigate whether these copy number imbalances reflect *AR* gene rearrangements that could be linked to splicing disruptions, we carried out a detailed analysis of *AR* gene structure in the LuCaP 86.2 and CWR-R1 models of CRPCa. By deletion-spanning PCR, we discovered a 8579-bp deletion of AR exons 5, 6 and 7 in the LuCaP 86.2 xenograft, which provides a rational explanation for synthesis of the truncated AR v567es AR variant in this model. Similarly, targeted resequencing of the *AR* gene in CWR-R1 cells led to the discovery of a 48-kb deletion in AR intron 1. This intragenic deletion marked a specific CWR-R1 cell population with enhanced expression of the truncated AR-V7/AR3 variant, a high level of androgen-independent AR transcriptional activity and rapid androgen independent growth. Together, these data demonstrate that structural alterations in the *AR* gene are linked to stable gain-of-function splicing alterations in CRPCa.

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INTRODUCTION

PCa initially presents as an androgen- and androgen receptor (AR)-dependent disease. Therefore, suppressing the production or action of androgens, which inhibits AR transcriptional activity, leads to stabilization or regression of advanced PCa. The duration of response to androgen depletion therapy (ADT) is variable with the end point typically marked by rising serum levels of prostate specific antigen, an AR transcriptional target gene and rapid growth of PCa metastases. This transition from androgen-dependent to castration-resistant prostate cancer (CRPCa) is frequently due to aberrant AR re-activation despite ongoing treatment with AR-targeted therapies.[1–4] Various mechanisms have been advanced to explain AR activation in CRPCa cells. These include *AR* gene amplification and/or AR protein over-expression,[5–12] point mutations that permit promiscuous AR transcriptional responses[13–20] and intra-tumor steroid synthesis or sequestration.[21–24] This knowledge has driven the clinical development of new inhibitors of androgen production and AR signaling,[25] including the CYP17 inhibitor abiraterone acetate, which has been recently shown to increase overall survival in patients with metastatic CRPCa.[26]

Synthesis of truncated AR variant proteins via AR alternative splicing has recently emerged as an additional mechanism of ADT resistance in PCa.[27–31] These proteins lack the AR ligand-binding domain, display constitutive, ligand-independent transcriptional activity, and mediate androgen-independent growth of PCa cells in various model systems.[27–31] Increased expression of the AR3 variant protein (also termed AR-V7[29]) in PCa prostatectomy specimens is associated with biochemical recurrence following surgery.[28] In addition, increased mRNA expression of alternatively spliced AR variants in PCa bone metastases is associated with shorter survival.[32] Therefore, understanding the mechanisms leading to increased synthesis of these species could provide important prognostic information, or perhaps guide more effective use of therapies that inhibit ligand-dependent AR activity.

Truncated AR variants proteins were originally discovered and functionally characterized in the CRPCa 22Rv1 and CWR-R1 cell lines,[27–29] and the LuCaP 86.2 PCa xenograft.[30] In 22Rv1 cells, a 35-kb AR intragenic tandem duplication is linked to altered splicing of full-length AR, as well as synthesis of truncated AR variants.[33] However, the mechanisms driving AR splicing

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alterations outside of the 22Rv1 model have remained elusive. Therefore, the purpose of this study was to investigate the link between *AR* gene structure alterations and enhanced synthesis of truncated AR variants in CRPCa.

RESULTS

AR gene structure complexity in CRPCa

In a previous study, we analyzed high-resolution whole-genome copy number data from CRPCa metastasis, which implicated frequent AR copy number imbalance concurrent with AR amplification at this stage of the disease.[33] To investigate this phenomenon directly, we employed a multiplex ligation-dependent probe assay (MLPA) with probe sets targeted to coding exons in the *AR* gene (Figure 1a). This MLPA approach detected the 22Rv1 duplication involving exon 3, as well as 20-fold amplification of the *AR* gene in VCaP cells (Figure 1b). Androgen-dependent PCa tissue obtained from xenografts (Figure 1c) or clinical specimens (Figure 1d) displayed one intact *AR* gene copy, with the exception of the LuCaP 35 xenograft, which displayed four copies of the *AR* gene. However, CRPCa tissue obtained from xenografts (Figure 1c) or autopsy specimens (Figure 1d) displayed frequent *AR* gene amplification and/or complex patterns of *AR* gene copy number imbalance. These data suggest that imbalances in *AR* gene copy number may be important for CRPCa progression.

To investigate this phenomenon in more detail, we focused on the LuCaP 86.2 xenograft, which expresses high levels of the truncated AR v567es variant arising from the mRNA splicing machinery skipping exons 5, 6 and 7.[30] LuCaP 86.2 displayed reduced genome copy number of these exons, indicating a mixed cell population with ~50% of cells harboring an intragenic deletion (Figure 1c). Deletion-spanning PCR yielded products consistent with an intragenic deletion encompassing AR exons 5, 6 and 7 (Figures 2a and b). Sequence analysis verified a 8579-bp deletion (Figure 2c) with microhomology at the 5′ and 3′ break fusion junctions, which signifies a non-homologous end joining mechanism of origin (Figure 2d). Together, these data implicate focal intragenic deletion as a novel mechanism underlying synthesis of the truncated AR v567es variant in the LuCaP 86.2 xenograft.

Stable AR mRNA splicing alterations in CRPCa CWR-R1 cells

MLPA analysis indicated that the CRPCa CWR-R1 cell line does not harbor any *AR* gene copy number alterations, despite previous studies demonstrating that these cells express truncated AR variants.[28] To confirm altered splicing in these cells, we assessed AR mRNA isoform levels using an absolute quantification reverse transcription (RT)–PCR assay. To correct for variability in *AR* gene dosage and rates of AR transcription, we scaled copy number for each AR mRNA isoform relative to full-length AR. This approach revealed changes in the ratios of full-length AR mRNA and alternatively spliced AR isoforms in CRPCa CWR-R1 and 22Rv1 cells (Figure 3b). Conversely, androgen-dependent CWR22Pc, LNCaP and VCaP cells expressed predominantly full-length AR mRNA (Figure 3b). To test for plasticity in the expression of truncated AR variant expression in CWR-R1 cells, we knocked down full-length AR mRNA using an AR exon 7-targeted siRNA. No changes in truncated AR protein expression were observed following 48 h or 72 h of knock down, whereas an AR exon 1-targeted siRNA completely abolished all AR protein expression in these cells (Figure 3c). Similarly, no changes in truncated AR variant protein expression were observed in CWR-R1 cells following 24 h or 72 h of androgen stimulation (Figure 3d). These data indicate that the altered AR mRNA splicing pattern in CWR-R1 cells is stable and is unlikely an acute cellular response to manipulations of androgen or AR levels.

A novel intragenic deletion in CWR-R1 cells identified by paired-end *AR* gene resequencing

As MLPA only interrogates AR copy number at coding exons, which represent less than 1.5% of the 180-kb *AR* gene, we

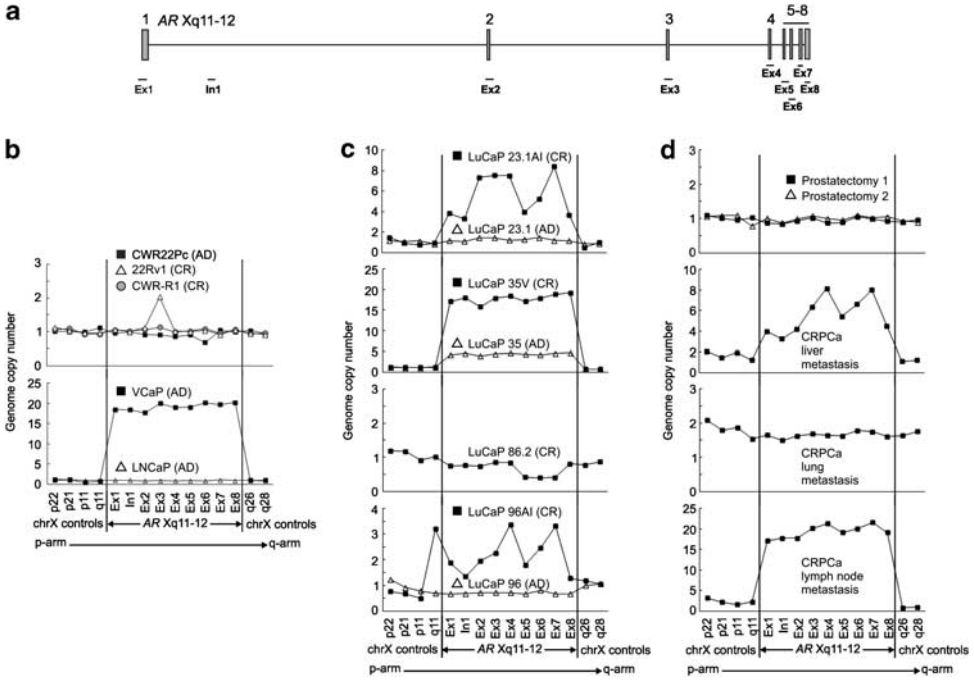

Figure 1. Diverse and complex patterns of *AR* gene copy number imbalance in CRPCa. (**a**) Schematic of the *AR* gene with relative locations of MLPA probes used for targeted copy number analysis. Genomic DNA from (**b**) androgen-dependent (AD) and castration-resistant (CR) PCa cell lines, (**c**) PCa xenografts, including AD/CR pairs propagated in intact/castrated male mice, and (**d**) clinical PCa was subjected to MLPA to evaluate genomic copy number across the AR locus and X chromosome.

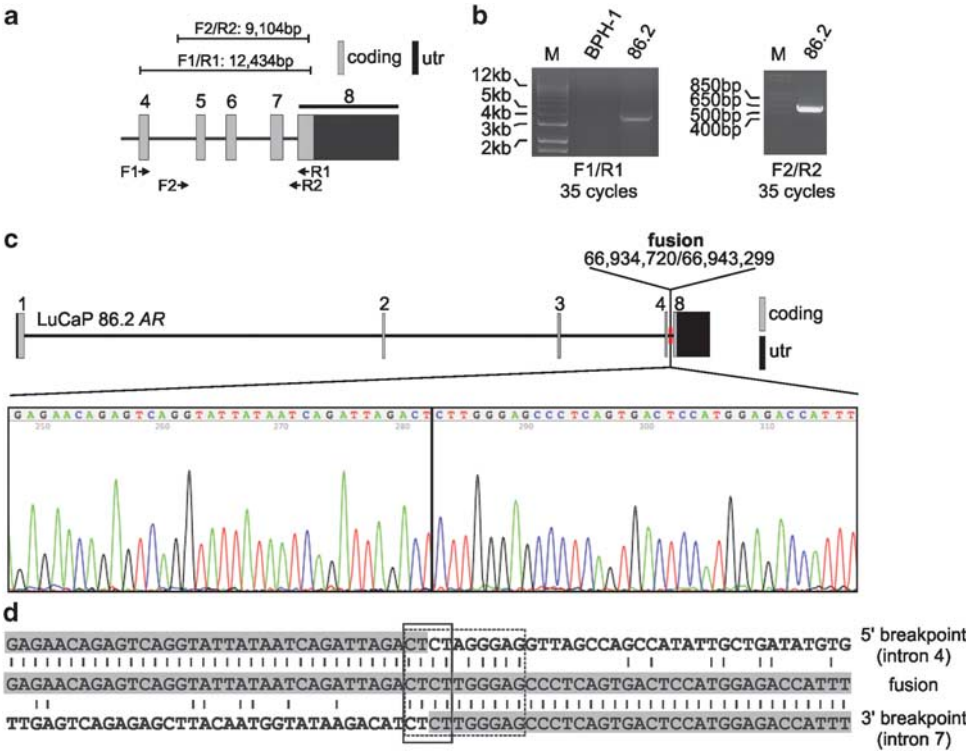

Figure 2. Intragenic deletion encompassing AR exons 5, 6 and 7 in the LuCaP 86.2 xenograft model. (**a**) Relative positions of deletion-spanning PCR primers and expected PCR fragment sizes based on the hg19 build of the human genome. (**b**) CWR-R1 and BPH-1 genomic DNA was subjected to nested PCR using primer sets indicated in (**a**). (**c**) PCR products from (**b**) were cloned and sequenced using the Sanger method. The electropherogram peak trace and *AR* gene structure resulting from the 8579-bp intragenic deletion are shown. (**d**) Alignment of the 5′-deletion breakpoint, the 3′-deletion breakpoint, and the deletion fusion revealed 4 bp of perfect microhomology (solid box) and 10 bp of extended microhomology with 1 bp mismatch (dashed box). Sequence retained in the break fusion junction is shaded in gray.

analyzed the nucleotide sequence and structure of the entire AR locus in CWR-R1 cells using a combination of liquid-phase sequence capture and Illumina paired-end massively parallel sequencing (Supplementary Figure 1 and Supplementary Table 1). Androgen-dependent CWR22Pc and CRPCa 22Rv1 cells were sequenced concurrently. As 49.9% of the AR locus is composed of repetitive DNA,[34] which precludes the design of capture baits to these regions, we were unable to obtain sequence for nearly a quarter of the *AR* gene using this strategy (Supplementary Table 1). In particular, no sequence was obtained within three 'gaps' of contiguous repetitive elements spanning >6 kb each (Figure 4 and Supplementary Figures 1 and 2). Gaps 2 and 3 harbor the 5′ and 3′ breakpoints of the previously identified 22Rv1 tandem duplication[33] and no paired-end reads representing this structural alteration were detected (Supplementary Table 2). However, sequence coverage plots revealed increased copy number within this region, with transition points located within gaps 2 and 3 (Supplementary Figures 2 and 3). No copy number imbalances in this region were observed in CWR22Pc or CWR-R1 cells (Supplementary Figure 2). Point mutations and indels were observed at various frequencies in all three cell lines, including the previously-identified H874Y coding mutation at position chrX:66 943 543 (Supplementary Tables 3–5). However, no prevalent sequence alterations were observed in splice donor/acceptor sequences in CWR-R1 and 22Rv1 cells (Supplementary Tables 4 and 5). Strikingly, structural variant analysis of paired-end reads using the Hydra workflow[35] identified a ~48-kb intragenic deletion within AR exon 1 in a sub-population of the CWR-R1 cell line, which was also apparent from a relative decrease in sequence coverage peak height within this region (Figure 4, Supplementary Table 2 and Supplementary Figure 4). To confirm this structural alteration in CWR-R1 cells, we performed nested PCR using primers spanning the deletion (Figures 5a and b). Sanger sequencing of cloned PCR products revealed deletion of 48 476 bp from AR intron 1 (Figures 5c and d). Alignments of the 5′ and 3′ break fusion junctions demonstrated 3 bp of microhomology, implicating non-homologous end joining as the mechanism underlying this deletion (Figure 5e).

To quantify the prevalence of this ~48-kb deletion, we performed MLPA with probe pairs custom-designed to query copy number at regular intervals along the length of the *AR* gene (Figure 6a). MLPA probe pairs targeted within this 48-kb region displayed a ~20–30% decrease in copy number (Figure 6b). There were no copy number alterations in this region detected by MLPA in CWR22Pc or 22Rv1 cells (Figure 6b), and nested PCR with deletion-spanning primers did not generate products in CWR22Pc or 22Rv1 cells (Figure 6c). Similarly, only CWR-R1 cells yielded 76 bp Illumina sequencing reads that could be aligned to a 130-bp template harboring this specific breakpoint sequence (Supplementary Figure 5). Together, these data demonstrate that a 48 476-bp deletion within intron 1 of the *AR* gene is restricted to 20–40% of the cells in the CWR-R1 cell line.

Enrichment for cells harboring AR intron 1 deletion during castration

The levels of truncated AR isoforms expressed in CWR-R1 cells are markedly higher when these cells are grown as xenografts in castrated mice versus intact mice.[28] All the analyses in our study had been performed with CWR-R1 cells that had been cultured in complete medium (contains androgens) for 10–20 passages, which would allow the growth of both androgen-dependent and

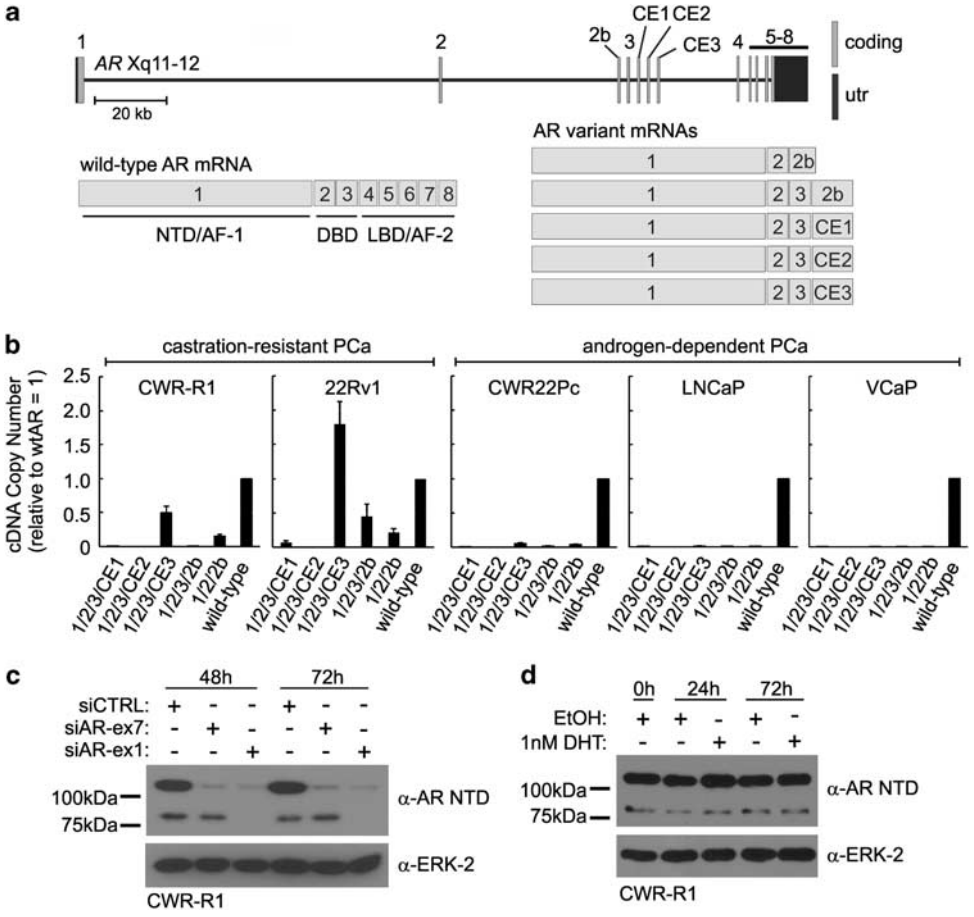

Figure 3. Stable, high-level expression of truncated AR variants in CWR-R1 cells. (**a**) AR genomic organization and exon composition of alternatively spliced AR mRNA isoforms reported in cell lines derived from the CWR22 xenograft. (**b**) RNA from indicated PCa cells lines was subjected to quantitative RT–PCR with isoform-specific primer sets, and Ct values were converted to copy number by plotting on standard curves. (**c**) CWR-R1 cells were electroporated with a control siRNA or siRNAs targeted to AR exon 1 or exon 7 and analyzed by western blot with indicated antibodies 48 h and 72 h post-transfection. (**d**) CWR-R1 cells were treated with 1 nM dihydrotestosterone (DHT) or vehicle (EtOH) for indicated times and analyzed by western blot with indicated antibodies.

CRPCa cell populations. Therefore, we compared *AR* gene structure in early passage CWR-R1 cells (referred to as CWR-R1 early) to CWR-R1 cells that were cultured in the absence of androgens for 20 passages (referred to as CWR-R1-late). Remarkably, no copy number decrease within AR intron 1 was apparent following MLPA analysis of CWR-R1 early cells (Figure 7a), despite a positive nested PCR signal for this deletion (Supplementary Figure 6). Conversely, MLPA probe signal in this region was nearly completely lost in CWR-R1 late cells (Figure 7a), indicating that this deletion was a marker of the CRPCa cell sub-population. Consistent with this deletion underlying the AR splicing patterns in CWR-R1 cells, expression of the truncated AR 1/2/3/CE3 variant (also referred to as AR-V7[29] or AR3[28]) was low in CWR-R1 early cells, regardless of whether they were cultured in whole serum or steroid-depleted serum (Figure 7b). However, CWR-R1 late cells displayed high-level expression of AR 1/2/3/CE3 protein (Figure 7b). These changes at the protein level corresponded with a stable shift in splicing favoring the AR 1/2/3/CE3 AR mRNA isoform in CWR-R1 late versus CWR-R1 early cells (Supplementary Figure 7). CWR-R1 cells cultured in the presence of androgens for 20 passages displayed an intermediate AR 1/2/3/CE3 protein expression pattern (Figure 7b). Immunostaining of cells grown under castrate conditions demonstrated increased nuclear expression of the AR NH_2-terminal domain versus the AR COOH-terminal domain in CWR-R1 late cells, but not CWR-R1 early cells (Supplementary Figure 8). Together, these data demonstrate that cells harboring this ~48-kb deletion within AR exon 1 display a splicing switch that favors stable, high-level expression of the truncated AR 1/2/3/CE3 variant.

To investigate whether there may be functional consequences to these differences in truncated AR variant expression, we examined AR transcriptional activity in CWR-R1 early and CWR-R1 late cells. AR transactivation in response to the synthetic androgen mibolerone was higher in CWR-R1 early cells than CWR-R1 late cells and knock down of full-length AR inhibited this androgen response in both cell lines (Figure 7c). Interestingly, knock down of AR expression with siRNAs targeted to either AR exon 1 or exon 7 inhibited androgen-independent transcriptional activity in CWR-R1 early cells, but only siRNA targeted to AR exon 1 had this effect in CWR-R1 late cells (Figure 7c). These data indicate that androgen independent AR activity in CWR-R1 early cells is dependent on full-length AR expression, whereas androgen-independent AR activity in CWR-R1 late cells is mediated by truncated AR variants through a mechanism that is independent of full-length AR. To investigate differential siRNA sensitivity and a differential role for full-length AR in more detail, we compared the androgen-independent growth of CWR-R1 early and CWR-R1 late cells transfected with AR-targeted siRNAs. Consistent with their selection under castrate conditions, CWR-R1 late cells displayed a rapid androgen-independent growth rate, which was inhibited by

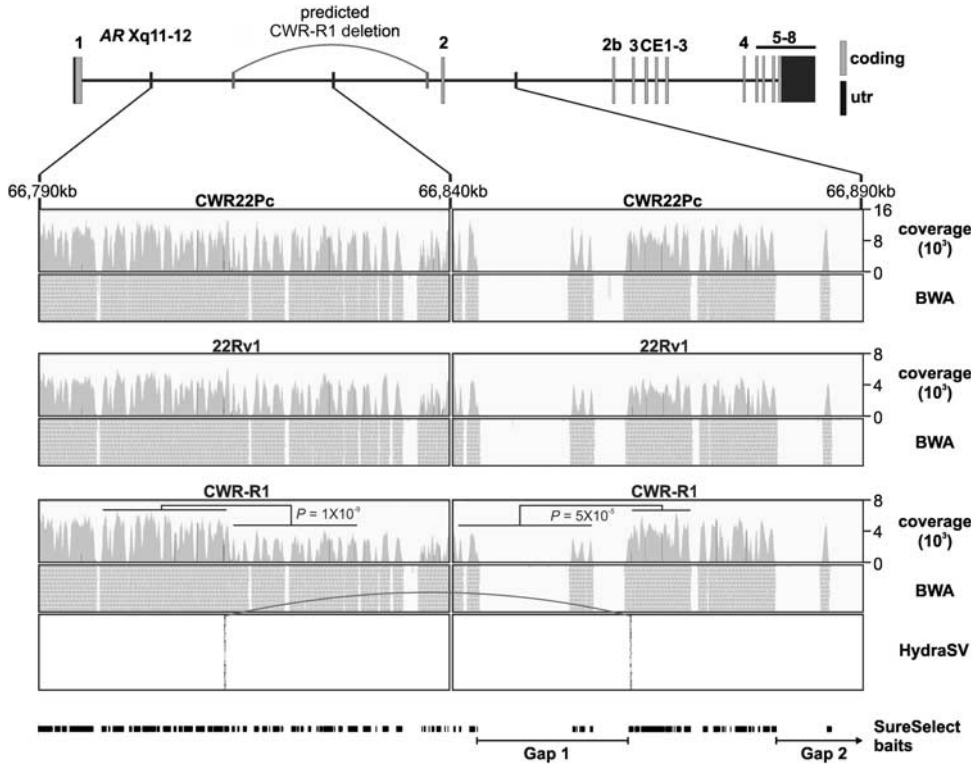

Figure 4. CWR-R1 cells harbor an intragenic deletion in AR intron 1. A schematic of the AR locus is illustrated at the top. Paired-end sequence reads were mapped to the hg19 build of the human genome using Burrows–Wheeler Alignment (BWA) and visualized using Integrated Genomics Viewer (IGV). Two 50-kb windows-spanning genomic positions 66 790 000 and 66 890 000 are shown for each cell line, indicating positions and depth of coverage of BWA-mapped reads. Discordantly mapped paired-end reads identified by the Hydra workflow are shown for CWR-R1 and the relative location of this predicted deletion is indicated on the AR locus schematic. Differences in coverage peak maxima at sites flanking the predicted deletion breakpoints were compared using *t*-tests. Positions of individual SureSelect baits used for AR sequence capture are tiled across the bottom. Two large gaps of extended repetitive sequence precluding design of capture baits are indicated.

siRNA targeted to AR exon 1, but not AR exon 7 (Figure 7d). Conversely, CWR-R1 early cells grew slowly under androgen-independent conditions during this short time-course and there was limited response to AR-targeted siRNA (Figure 6d).

DISCUSSION

The prevalence of *AR* gene mutations in CRPCa reported by different groups has been variable, but appears to be low at approximately 10%.[13–20] These data suggest that the majority of CRPCa harbors a wild-type *AR* gene. However, mutations have been evaluated historically by sequence analysis of AR cDNA and/or AR coding exons, which represents only ~1.5% of the 180 245-bp *AR* gene.[36] The identification and clinical validation of truncated AR variant synthesis as an important mechanism of PCa therapy resistance has raised the possibility that splicing disruptions may be due to non-coding *AR* gene alterations. Indeed, our previous work defined a ~35-kb AR intragenic tandem duplication in 22Rv1 cells, which is a rational explanation for the altered splicing pattern in this cell line.[33] In this study, we have defined two additional simple *AR* gene structural alterations that are linked to the pathological AR splicing patterns in the LuCaP 86.2 and CWR-R1 models of PCa progression. These data, combined with MLPA analysis of additional CRPCa specimens, indicate that the prevalence of *AR* gene alterations in tumors resistant to ADT may be higher than previously anticipated. Although targeted methods such as MLPA are useful for identifying deletions or duplications that involve probe-binding sites, this study has illustrated that unbiased evaluation of the entire *AR* gene sequence and structure is a preferable approach. However, our work has also demonstrated that high-throughput approaches are challenged by substantial repeat in the AR locus. Indeed, paired-end reads diagnostic of the 35-kb 22Rv1 AR intragenic tandem duplication were not obtained in our study, despite 6000-fold maximal on-target sequence coverage in these cells. This indicates that many *AR* gene structural alterations would also go undetected using larger-scale sequence capture or whole-genome sequencing approaches.[37,38] Therefore, to define the prevalence and spectrum of the *AR* gene structure alterations that may exist in clinical CRPCa, methodology optimization must be a prerequisite.

Previous analysis of genome-wide copy number data from clinical CRPCa specimens suggested that complex patterns of copy number gain and copy number loss occurred along the length of the *AR* gene,[33] which is supported by MLPA analysis in this study. Here, we demonstrated that large deletions involving intron 1 are associated with enhanced synthesis of the truncated AR 1/2/3/CE3 variant (also referred to as AR-V7 or AR3) and a growth advantage under castrate conditions. In the CWR-R1 model, castration-mediated enrichment for cells harboring intron 1 deletion resulted in an overall population that exhibited levels of AR 1/2/3/CE3 that were equivalent to or greater than the levels of full-length AR. This is important because a recent study of surgical specimens of CRPCa bone metastases with an antibody specific for the AR NTD demonstrated that protein expression of truncated AR variants can reach similar high levels relative to full-length AR.[32] Moreover, patients with CRPCa bone metastases that displayed the highest levels of alternatively spliced, truncated AR mRNA variants had shorter cancer-specific survival after metastasis

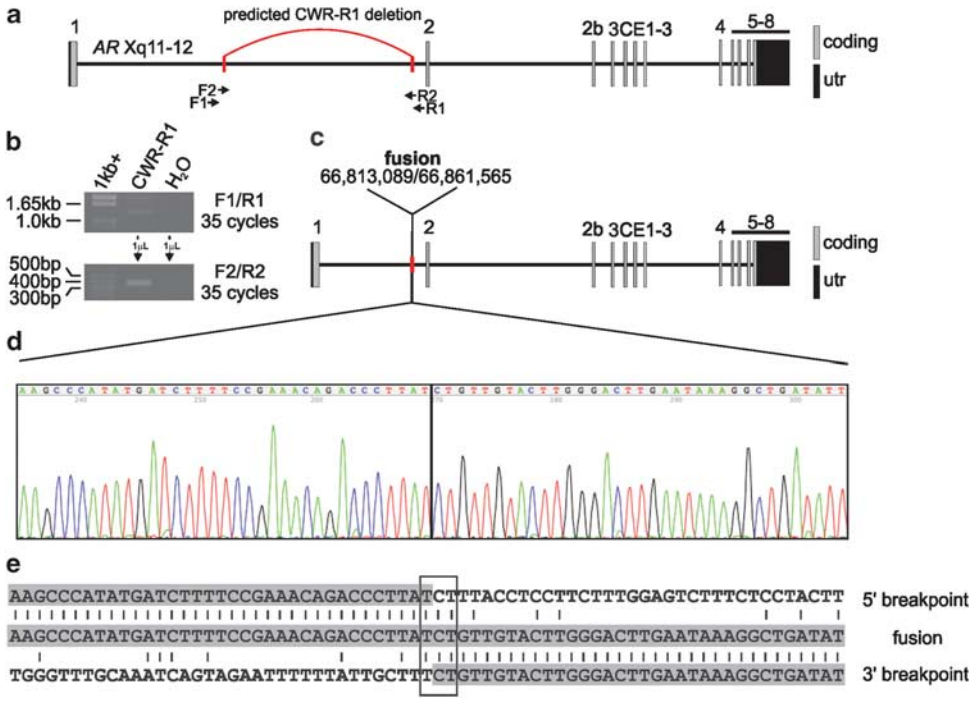

Figure 5. Intragenic deletion involving 48 476 bp of AR intron 1 in CWR-R1 cells. (**a**) Schematic of the AR locus with relative positions of deletion-spanning PCR primers. (**b**) CWR-R1 genomic DNA was subjected to nested PCR using indicated primer sets indicated in (**a**). (**c**) Schematic of the CWR-R1 AR locus harboring a 48 476-bp intron 1 deletion. (**d**) PCR products from (b) were cloned and sequenced using the Sanger method. The electropherogram peak trace is shown. (**e**) Alignment of the 5′-deletion breakpoint, the 3′-deletion breakpoint, and the deletion fusion revealed 3 bp of microhomology (boxed). Sequence retained in the break fusion junction is shaded in gray.

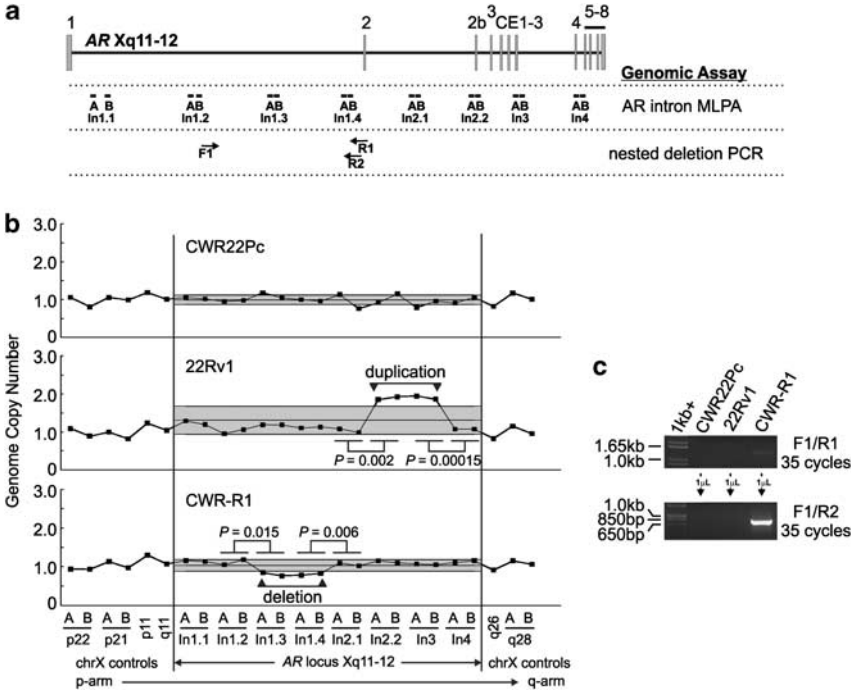

Figure 6. Intragenic deletion is restricted to a CWR-R1 subpopulation. (**a**) Schematic of the *AR* gene with relative locations of primers and probes used for targeted genomic assays. (**b**) Genomic DNA was subjected to MLPA to evaluate genomic copy number across the AR locus and X chromosome. Relative positions of AR-specific MLPA probes are indicated in (**a**). Gray boxes represent the mean ± s.d. of all AR locus probes from two independent experiments. Probe pairs displaying copy number greater than one s.d. away from the mean AR copy number are diagnostic of duplication or deletion. Differences in copy number measured by probe pairs flanking predicted duplication or deletion breakpoints were compared with *t*-tests. (**c**) Genomic DNA was subjected to nested PCR using primer sets depicted in (**a**).

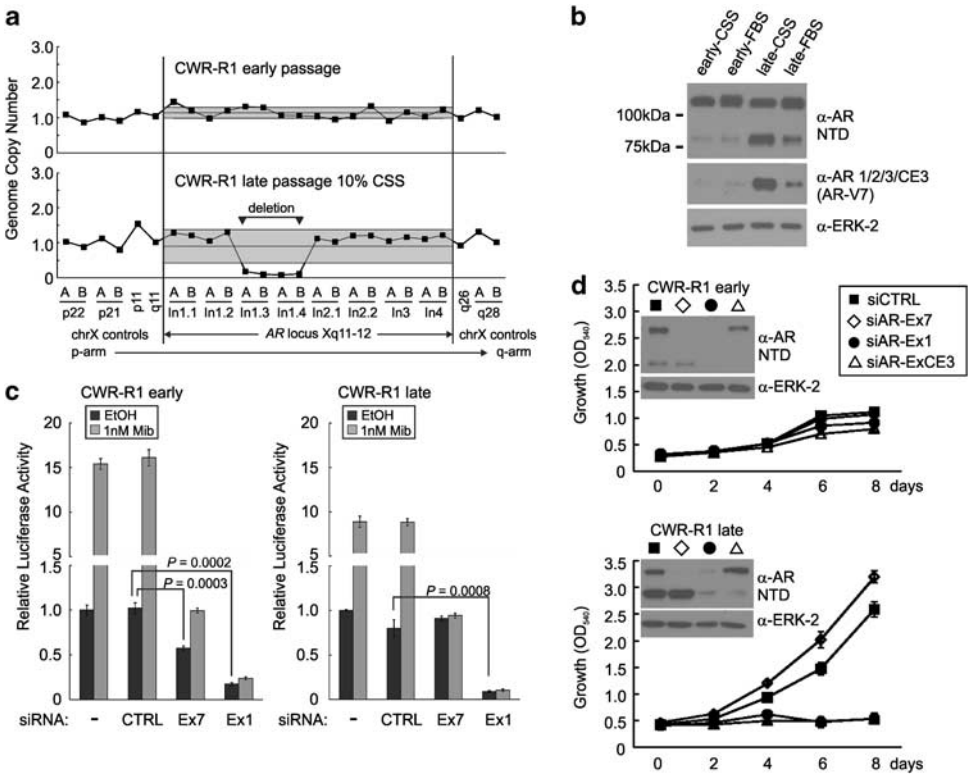

Figure 7. CWR-R1 cells with intron 1 deletion are castration-resistant and dependent on truncated AR variant function. (**a**) Genomic DNA from early-passage CWR-R1 cells and CWR-R1 cells cultured for 20 passages under castrate conditions was subjected to MLPA to evaluate genomic copy number across the AR locus and X chromosome. Gray boxes represent the mean ± s.d. of all AR locus probes from two independent experiments. Probe pairs diagnostic of deletion are indicated. (**b**) Lysates from early-passage CWR-R1 cells cultured in the presence (fetal bovine serum (FBS)) or absence (charcoal-stripped serum (CSS)) of androgens for 24 h and CWR-R1 cells cultured in the presence (FBS) or absence (CSS) of androgens for 20 passages (late) were analyzed by western blot with indicated antibodies. (**c**) Early-passage CWR-R1 cells and CWR-R1 cells cultured for 20 passages under castrate conditions (late) were transfected with MMTV-Luc, non-targeted control (CTRL) siRNA, or siRNAs targeted to AR exon 1 or 7. Cells were grown 24 h in serum-free medium and treated with 1 nM mibolerone (synthetic androgen) or EtOH (vehicle control) for 24 h. Luciferase activity was determined. Data represent the mean ± s.e. from two independent experiments, each performed in duplicate. MMTV promoter activity without androgens and siRNAs was arbitrarily set to 1. (**d**) Early-passage CWR-R1 cells and CWR-R1 cells cultured for 20 passages under castrate conditions (late) were transfected with non-targeted CTRL siRNA, or siRNAs targeted to AR exon 1 or 7. Growth of transfected cells under castrate conditions was monitored every 2 days.

surgery than other CRPCa patients.[32] Therefore, increased expression of truncated AR variants is an important component of clinical PCa progression. The data in this study strongly suggests that alterations in the architecture of the *AR* gene may underlie these disruptions in normal splicing patterns. With this in mind, it is important to point out that a true 'alternative splicing' mechanism has not yet been elucidated for the *AR* gene. Rather, models that exhibit levels of truncated AR variant expression sufficient to drive the CRPCa phentoype (such as 22Rv1, CWR-R1 and LuCaP 86.2) have dramatic changes in the *AR* gene template. These AR intragenic duplications and deletions that we have defined all appear to result from homologous recombination-independent mechanisms such as microhomology-mediated break-induced replication in 22Rv1[33] and non-homologous end joining in LuCaP 86.2 and CWR-R1. Therefore, the exact locations of breakpoints in the AR locus are unlikely to be recurrent between specimens. Intriguingly, these three models each display a unique splicing signature and repertoire of truncated AR variant protein expression. Therefore, it is tempting to speculate that different patterns of *AR* gene alteration may give rise to different AR splicing patterns in clinical CRPCa. This would argue that a complete understanding of the role of truncated AR variants in CRPCa progression will require that individual tumors be evaluated for splicing alterations using unbiased detection methods rather than targeted approaches focused on known AR variants.

Overexpression of the AR 1/2/3/CE3 variant (also referred to as AR-V7 or AR3), the AR v567es variant, or a truncated AR variant of mouse origin (mAR-V4) in LNCaP cells can induce androgen-independent expression of AR target genes and growth under castrate conditions *in vitro* and *in vivo*.[28,30,31] Interestingly, treatment of these engineered LNCaP cells with the next-generation anti-androgen MDV3100 or knock down of full-length AR resulted in reversal of these CRPCa features.[31] These data indicate that truncated AR variants require full-length AR to support a CRPCa phenotype. However, this is in opposition to our studies with CRPCa models that endogenously express high levels of truncated AR variants and harbor apparent gain-of-function structural alterations in the *AR* gene.[27,33] For example, in this study, knock down of full-length AR had no effect on androgen-independent AR activity or androgen-independent growth in late-passage CWR-R1 cells. However, knock down of AR 1/2/3/CE3 inhibited these parameters. We have also demonstrated this differential response to isoform-targeted siRNAs in the 22Rv1 cell line.[27] Conversely, early-passage CWR-R1 cells displayed modest androgen-independent growth and measurable androgen-independent AR activity, which was inhibited following knock down of full-length AR. These data demonstrate that the CWR-R1 cell line is heterogeneous and that growth conditions can have dramatic effects on the relative proportions of androgen-dependent cells and CRPCa cells, which may explain a previous report where

CWR-R1 cells displayed decreased proliferation and increased apoptosis in response to full-length AR knockdown.[28] With this in mind, it is also important to note that the LuCaP 86.2 xenograft tissue evaluated in this study was propagated in an intact male mouse, and MLPA data reflected an approximate 50/50 mixture of cells with either one intact *AR* gene copy or one *AR* gene copy with a 8579-bp deletion of exons 5, 6 and 7. If the cell population harboring the 8549-bp intragenic deletion is indeed the cell population which synthesizes the AR v567es variant, these cells would not be able to synthesize full-length AR and would be truly independent of full-length AR activity.[30] Therefore, a more thorough investigation of the requirement for full-length AR is warranted, as this will provide important insights to resistance mechanisms that may circumvent clinical responses to current and next-generation therapies targeting the AR ligand-binding domain.[25]

In summary, this study represents the first report of intragenic deletions involving coding and non-coding sequences in the *AR* gene in CRPCa, which we have linked to expression of truncated AR variants that support the CRPCa phenotype. Therefore, structural alterations in the *AR* gene may represent a widespread, yet previously unanticipated, mechanism of therapy resistance in PCa. Our findings provide justification for large-scale investigation of *AR* gene structure and splicing patterns in clinical specimens.

MATERIALS AND METHODS

PCa tissues

Genomic DNA samples from the LuCaP series of PCa xenografts and de-identified clinical CRPCa tissue were obtained from the University of Washington Prostate Cancer Biorepository, which was developed and managed by one of the co-authors (RLV) and has been described in previous publications.[30,39,40] De-identified prostatectomy tissue samples were obtained under the direction of the University of Minnesota BioNet tissue resource, which was developed and managed by one of the co-authors (SCS). Cores of PCa tissue (1 mm) were obtained from archival formalin-fixed, paraffin-embedded prostatectomy blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA) and genomic DNA was isolated using a RecoverAll kit (Applied Biosystems/Ambion, Austin, TX, USA).

Cell culture

The 22Rv1 (number CRL-2505), LNCaP (number CRL-1740) and VCaP (number CRL-2876) cell lines were obtained from ATCC (Manassas, VA, USA) and cultured according to ATCC protocol. CWR22Pc cells[41] were generously provided by Dr Marja Nevalainen (Thomas Jefferson University) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2.5 mM L-glutamine and 0.8 nM dihydrotestosterone (Sigma, St Louis, MO, USA). CWR-R1 cells[42] were a kind gift from Dr Elizabeth Wilson (UNC Chapel Hill) and cultured in RPMI 1640 + 10% fetal bovine serum. For androgen response experiments, cells were cultured in RPMI 1640 + 10% steroid-depleted, charcoal-stripped serum for 48 h, treated at $t = 0$ with 1 nM dihydrotestosterone (Sigma) or vehicle (EtOH), and then harvested at indicated time points. For long-term culture experiments, CWR-R1 cells were cultured in RPMI 1640 + 10% charcoal-stripped serum. Cells were trypsinized and re-seeded in the appropriate medium when flasks attained 80% confluence.

Transient transfections

The CWR-R1 cell line was electroporated with siRNAs targeted to AR exon 7 (target sequence: 5′-GGAACUCGAUCGUAUCAUU-3′) or AR exon 1 (5′-CAAG GGAGGUUACACCAAA-3′) and/or an MMTV-LUC reporter as described.[27] Growth of electroporated cells was monitored by crystal violet staining as described.[33] Luciferase activity was measured as described.[27]

Quantitative real-time RT–PCR. RNA isolation and absolute quantification RT–PCR analysis of alternatively-spliced AR mRNA isoforms was performed as described.[33] To correct for different levels of wild-type AR mRNA expression among the PCa cell lines, copy numbers of AR mRNA isoforms were scaled relative to wild-type AR mRNA copy number in each cell line (set to 1). For relative quantification RT–PCR, fold change in expression levels were determined by the comparative Ct method using the equation $2^{-\Delta\Delta Ct}$.

Genomic PCR. Genomic PCR was performed as described,[33] using primer pairs listed in Supplementary Table 6.

Western blot

Western blotting with AR NTD (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), AR CTD (Santa Cruz C-19), ERK-2 (Santa Cruz D-2) and ARV-7 (number AG10008, Precision Antibody, Columbia, MD, USA) antibodies was performed as described.[33]

Multiplex ligation-dependent probe assay

MLPA for AR coding sequence was performed using a commercially available kit (P074, MRC Holland, Amsterdam, The Netherlands) as per the manufacturer's protocol. Briefly, 100 ng of genomic DNA was hybridized at 60 °C for 18 h with MLPA probemix. Hybridized probes were ligated and amplified by PCR with labeled universal primers provided with the MLPA kit. PCR reactions were diluted 1:10 in formamide containing ROX-500 size standards (Applied Biosystems, Carlsbad, CA, USA), denatured and resolved by capillary electrophoresis using a Genetic Analyzer 3130XL (Applied Biosystems). Electropherogram peak areas were obtained using Peak Scanner software (Applied Biosystems). Peak areas for samples and calibration control (HPV-7 prostate epithelial cell genomic DNA) were block-normalized using X-chromosome p-arm controls and then normalized to HPV-7 copy number with the inference that the HPV-7 genome contains one copy of the *AR* gene. MLPA for AR intron sequences was performed using the exact same protocol with a commercially available reagent kit (EK1, MRC Holland) and custom-designed oligonucleotide probes (Supplementary Table 7). Probe pairs that each displayed copy number values outside of one s.d. from the mean copy number of all AR locus probes from two independent experiments were determined to have increased or decreased copy number at that location.

Paired-end library creation, sequence capture and next-generation sequencing

Genomic DNA from CWR22Pc, 22Rv1 and CWR-R1 cells was fragmented using an S220 ultra-sonicator (Covaris, Woburn, MA, USA) with Agilent SureSelect parameters. A Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, CA, USA) was used to verify DNA samples sheared with fragment peaks between 150–200 bp. Paired-end sequencing libraries were generated from sheared DNA samples using a SureSelect Library Preparation Kit (Agilent) and amplified for sequence capture as per the manufacturer's protocol. The amplified DNA libraries were hybridized and captured using overlapping, tiled SureSelect baits (Agilent) custom-designed to provide two times coverage of non-repetitive regions of the AR locus (Supplementary Table 8). Target-enriched libraries were amplified for 16-cycles to add index tags and generate sufficient template for flowcell clustering. Final libraries were quantified via quantitative PCR (Kapa Biosystems, Woburn, MA, USA), normalized and pooled before clustering on a single lane of a flowcell. The flowcell was loaded on a Genome Analyzer IIx (GAIIx, Illumina, San Diego, CA, USA) for paired-end sequencing at 76 cycles (2×76 bp). Data analysis methodology is provided as Supplemental Information.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Androgen Receptor Splice Variants Activate Androgen Receptor Target Genes and Support Aberrant Prostate Cancer Cell Growth Independent of Canonical Androgen Receptor Nuclear Localization Signal*[S]

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Background: Truncated AR splice variants support castration-resistant prostate cancer.
Results: The AR NTD/DBD core is sufficient for AR variants to access the nucleus, activate AR target genes, and support androgen-independent prostate cancer cell growth.
Conclusion: Diverse truncated AR variants are constitutively active transcription factors.
Significance: These novel biochemical properties could lead to the development of new prostate cancer therapies.

Synthesis of truncated androgen receptor (AR) splice variants has emerged as an important mechanism of prostate cancer (PCa) resistance to AR-targeted therapy and progression to a lethal castration-resistant phenotype. However, the precise role of these factors at this stage of the disease is not clear due to loss of multiple COOH-terminal AR protein domains, including the canonical nuclear localization signal (NLS) in the AR hinge region. Despite loss of this NLS, we show that diverse truncated AR variant species have a basal level of nuclear localization sufficient for ligand-independent transcriptional activity. Whereas full-length AR requires Hsp90 and importin-β for active nuclear translocation, basal nuclear localization of truncated AR variants is independent of these classical signals. For a subset of truncated AR variants, this basal level of nuclear import can be augmented by unique COOH-terminal sequences that reconstitute classical AR NLS activity. However, this property is separable from ligand-independent transcriptional activity. Therefore, the AR splice variant core consisting of the AR NH_2-terminal domain and DNA binding domain is sufficient for nuclear localization and androgen-independent transcriptional activation of endogenous AR target genes. Indeed, we show that truncated AR variants with nuclear as well as nuclear/cytoplasmic localization patterns can drive androgen-independent growth of PCa cells. Together, our data demonstrate that diverse truncated AR species with varying efficiencies of nuclear localization can contribute to castration-resistant PCa pathology by driving persistent ligand-independent AR transcriptional activity.

The androgen receptor (AR)[2] is a modular steroid receptor transcription factor, composed of a large, disordered NH_2-terminal domain (NTD), a central DNA binding domain (DBD), and a COOH-terminal ligand binding domain (LBD) and transcriptional activation function-2 coactivator binding interface (1). Androgen depletion therapy (ADT) for locally advanced or metastatic prostate cancer (PCa) relies on blocking androgen production and/or androgen binding to the AR LBD. However, most PCa mortality is due to the development of castration-resistant PCa (CRPCa), which is driven by pathologic reactivation of the AR during ADT (2). An ongoing AR dependence of CRPCa supports the concept that AR is a lineage survival factor in prostate cells (3), and this knowledge has led to the development of additional strategies for AR inhibition, including the CYP17 inhibitor abiraterone, which extends lifespan in a subset of CRPCa patients by further suppressing androgen production in the adrenal cortex as well as tumor tissue (4).

One possible mechanism of aberrant AR reactivation during ADT is the synthesis of COOH-terminal truncated AR variants lacking the AR LBD and activation function-2 domain (5). These AR variants arise through alterations in splicing and encode protein products composed of the AR NTD/DBD core and novel COOH-terminal extensions of variable length and sequence (6–10). Antibodies have been developed for the COOH-terminal extension of the truncated AR-V7 variant (also referred to as AR3), demonstrating that expression levels increase concomitant with stage of hormonal progression (7). Moreover, increased expression of AR-V7 in hormone-naïve prostatectomy specimens is associated with disease relapse

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[S] This article contains supplemental Experimental Procedures, Table 1, and Figs. 1–8.

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[2] The abbreviations used are: AR, androgen receptor; NTD, NH_2-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain; ADT, androgen depletion therapy; PCa, prostate cancer; CRPCa, castration-resistant PCa; NLS, nuclear localization signal; ARE, androgen response element; LUC, luciferase.

after surgery (7). In surgical specimens of skeletal CRPCa metastases, levels of AR-V7 and AR-V1 mRNA are inversely correlated with survival (11). These studies indicate that changes in AR splicing are important for clinical CRPCa progression and may drive resistance to therapies that require an intact AR LBD, including next-generation inhibitors such as abiraterone (12).

Currently, it is unclear whether diverse truncated AR variants can activate the AR transcriptional program because many of these species are predicted to have lost the canonical AR nuclear localization signal (NLS) in the exon 4-encoded hinge region. Similar to other steroid receptors, AR transcriptional activity is tightly regulated by ligand binding. In the unbound, inactive state, the AR is a predominantly cytoplasmic protein bound by heat shock family chaperone proteins and immunophilins (13). AR nuclear import proceeds via a classical pathway wherein nuclear translocation is initiated by ligand binding, which exposes the bipartite AR NLS and allows binding to the importin-α adapter protein and importin-β carrier protein, translocation through the nuclear core complex, and Ran-dependent release into the nucleus (14, 15). Because domains required for interaction with these pathways appear to be lost, it has been suggested that many truncated AR variants may have exclusively cytoplasmic functions or even function as dominant negative species (7, 9, 10). Therefore, how these diverse species may be involved in CRPCa progression is not clear. To address this, we investigated the biochemical properties of individual truncated AR variants with the goal of identifying general molecular features that could be exploited for developing new CRPCa therapies.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—LNCaP, 22Rv1, DU145, 293T, and Cos-7 cells were obtained from American Type Culture Collection (ATCC). LNCaP, 22Rv1, and DU145 cells were maintained in RMPI 1640 (Invitrogen) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37 °C. The 293T and Cos-7 cell lines were cultured in DMEM with 10% FBS. For androgen response experiments, cells were seeded in 10% charcoal-stripped serum for 48 h. Cells were then stimulated for 24 h by replacement of growth medium with medium containing 1 nM mibolerone (Biomol) or dihydrotestosterone (Sigma).

Antibodies and siRNA Reagents—Small interfering RNAs (targeted to AR exons 1, 3, and 7) were purchased from Dharmacon. Primary antibodies specific for the AR NTD (N-20, catalogue #sc-816; A-441, catalogue #sc-7305), ERK-2 (D-2, catalogue #sc-1647), HA tag (F-7, catalogue #sc-7392), and α-tubulin (catalogue #sc-23948) were purchased from Santa Cruz Biotechnology. An antibody specific for lamin A/C (4C11, catalogue #4777) was purchased from Cell Signaling.

Plasmids—Plasmid constructs harboring full-length AR (p5HBhAR-A), AR 1/2/3/2b, MMTV-LUC, −5746 PSA-LUC, and 4XARE-E4-LUC have been described (6, 16). Construction details for plasmid and lentiviral reagents used in this study are provided in the supplemental Experimental Procedures.

Luciferase Reporter Gene Assays—LNCaP cells were transfected by electroporation exactly as described (6, 17). DU145 cells were transfected using Superfect reagent (Qiagen) exactly as described (6). For all reporter-based experiments, 24–48 h post-transfection cells were re-fed with serum-free medium containing 1 nM mibolerone or 0.1% ethanol as vehicle control for 24 h. Cells were harvested in 1× passive lysis buffer provided in a Dual Luciferase Assay kit (Promega). Activities of the firefly and *Renilla* luciferase reporters were assayed using a Dual Luciferase Assay kit as per the manufacturer's recommendations. Transfection efficiency was normalized by dividing firefly luciferase activity by *Renilla* luciferase activity. Data presented represent the mean ± S.E. from at least three independent experiments, each performed in triplicate.

Subcellular Fractionation—LNCaP cells were transfected under androgen-free conditions with expression vectors encoding truncated AR variants using PolyExpress (Excellgen) exactly as per the manufacturer's protocol. 22Rv1 cells were electroporated under androgen-free conditions with siRNA targeted to AR exon 7 (Dharmacon) as described (6). Transfected cells were cultured 24 h post-transfection and then treated for 24 h with medium containing 1 nM mibolerone or 0.1% ethanol. After treatment, transfected cells were washed in 1× phosphate-buffered saline (PBS), harvested in hypotonic buffer (250 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and a 1× final concentration of Roche Applied Science Mini complete protease inhibitor), and incubated for 20 min on ice. Cells were lysed by 10 passages through 25-gauge needles. The cytosolic fraction (supernatant) was collected by centrifugation at 720 × *g*, 5 min, 4 °C. The nuclear pellet was washed twice by resuspending in 500 μl of hypotonic buffer followed by 10 passages through 25-gauge needles. Nuclei were pelleted by centrifugation at 3000 × *g* for 10 min at 4 °C. Isolated cytosolic and nuclear fractions were resuspended in 1× Laemmli buffer (18), boiled for 5 min, and then loaded equally by volume for Western blot analysis.

Western Blot—Cell lysates in 1× Laemmli buffer were subjected to Western blot analysis as described (6). Blots were incubated with primary antibodies overnight at 4 °C and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Blots were developed by incubation with Super Signal chemiluminescence reagent (Pierce) and exposed to film.

Immunofluorescence—Cos-7, LNCaP, and DU145 cells were seeded in complete medium on coverslips the day before transfection. Cells were transfected under androgen-free conditions with 1 μg of expression vector encoding full-length AR or truncated AR variants using Lipofectamine 2000 (Invitrogen) for Cos-7 cells, electroporation for LNCaP cells, or Superfect (Qiagen) for DU145 cells. The next day culture medium was replaced with serum-free medium containing 1 nM mibolerone or 0.1% ethanol. Cells were maintained an additional 24 h and then fixed with ice-cold methanol for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, and blocked with 1× PBS containing 10% FBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with AR-N20 diluted 1:1000 or HA-F7 diluted 1:500 in PBS. Unbound primary antibody was removed by three washes with PBS, and then cells were incubated with fluorescein isothiocya-

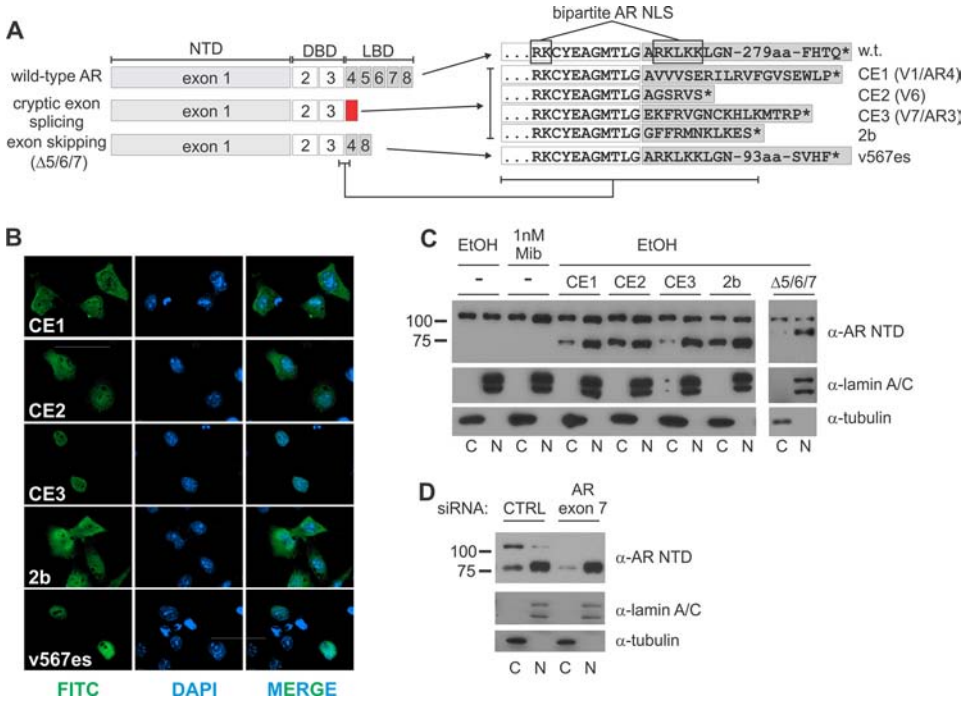


FIGURE 1. **Constitutive nuclear localization of truncated AR variants with diverse COOH-terminal extensions.** *A*, shown is a schematic of COOH-terminal tails of truncated AR variants aligned with the AR hinge region. The multiple names that have been assigned to several of these variants are indicated (7, 8). *B*, Cos-7 cells expressing truncated AR variants 1/2/3/CE1, 1/2/3/CE2, 1/2/3/CE3, 1/2/3/2b, or v567es were stained with an antibody specific for the AR NTD (FITC signal), and nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy. Representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *C*, LNCaP cells expressing truncated AR variants were cultured in serum-free medium containing 1 nM mibolerone (*Mib*, synthetic androgen) or ethanol (*EtOH*, vehicle control). Cell lysates were separated into nuclear (*N*) and cytoplasmic (*C*) fractions and analyzed by Western blot with antibodies specific for the AR NTD, lamin A/C (nuclear marker), and tubulin (cytoplasmic marker). *D*, 22Rv1 cells were electroporated with non-targeted siRNA (*CTRL*) or siRNA targeted to AR Exon 7. Cells were cultured, fractionated, and analyzed by Western blot exactly as described in *C*.

nate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) at a 1:500 dilution in PBS containing 0.2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 1 h at room temperature. Slides were washed with PBS and mounted with Mowoil (Calbiochem), and images were captured using a confocal laser scanning microscope (Olympus Fluoview FV500) equipped with a 40× objective. To optimize microscopy data for print media, hue adjustments were made to all immunofluorescence images (*red*, +90; *green*, +90; *blue*, −90) using CorelDraw software.

Lentivirus Packaging and Transduction—Lentivirus expressing GFP, AR 1/2/3/CE3, 1/2/3/CE3-R617A/K618A, 1/2/3/CE3-K629A/R631A, AR 1–627aa, AR 1/2/3/2b, and AR v567es was prepared using a standard third generation packaging system in 293T cells. Briefly, 293T cells were cotransfected with lentivirus vectors and packaging vectors pCMVΔR8.91 (19) and pMD.G (20) at a ratio of 4:3:1 using Lipofectamine 2000. Medium containing lentivirus was collected from 12 to 96 h post-transfection and concentrated using the Lenti-X Concentrator (Clontech). Titers for LNCaP transductions were tested and normalized to achieve protein expression levels equivalent to endogenous AR.

Total RNA Extraction and Quantitative Real Time RT-PCR—Total RNA was isolated from LNCaP cells using TRIzol (Invitrogen). RNA was reverse-transcribed using a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science) following the manufacturer's instructions. cDNA was subjected to quantitative PCR using PerfeCTa SYBR Green FastMix (Quanta Biosciences) with primer sets specific for PSA, hK2, TMPRSS2, FKBP51, and GAPDH (see supplemental Table 1). -Fold change in mRNA expression levels was calculated by the comparative Ct method using the formula $2^{-(\Delta\Delta Ct)}$ and GAPDH as calibrator as described (6).

Analysis of Cell Growth by Crystal Violet Mitogenic Assay—Lentivirus-transduced LNCaP cells were seeded at equal density on 24-well plates. Lentivirus-transduced 22Rv1 cells were electroporated with small interference RNA (siRNA) targeted to AR exon 1 as described (6, 17) and seeded at equal density on 24-well plates. At the indicated time points, cells were fixed and stained with crystal violet as described (21).

RESULTS

Truncated AR Variants with Diverse COOH-terminal Extensions Are Constitutively Nuclear Transcription Factors—The majority of AR transcriptional activity is mediated by the AR NTD and deletion of the AR LBD results in constitutive, ligand-independent activity (22). However, early biochemical studies that established these fundamental principles employed AR fragments that retained the bipartite NLS in the AR hinge region. Most truncated AR variants expressed in CRPCa have a disrupted bipartite NLS due to altered splicing (Fig. 1*A*). Therefore, it has been proposed that many of these species would not be able to access the nucleus, which is a prerequisite for transcriptional activation. However, this theory has not been rigorously tested. Therefore, we transfected cells with diverse truncated AR variants and performed immunofluorescence

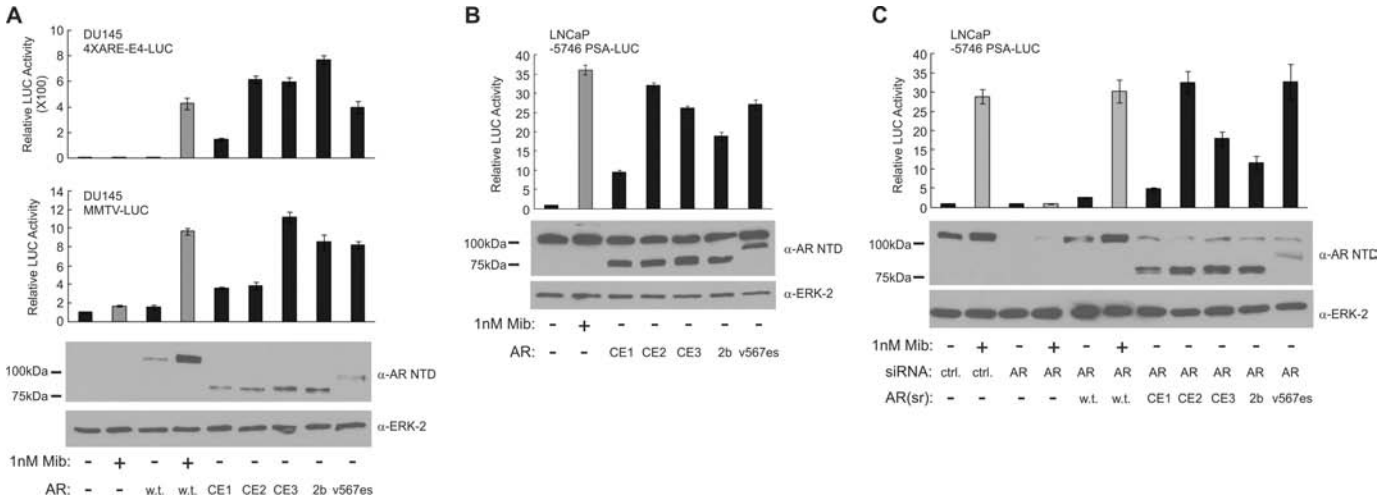


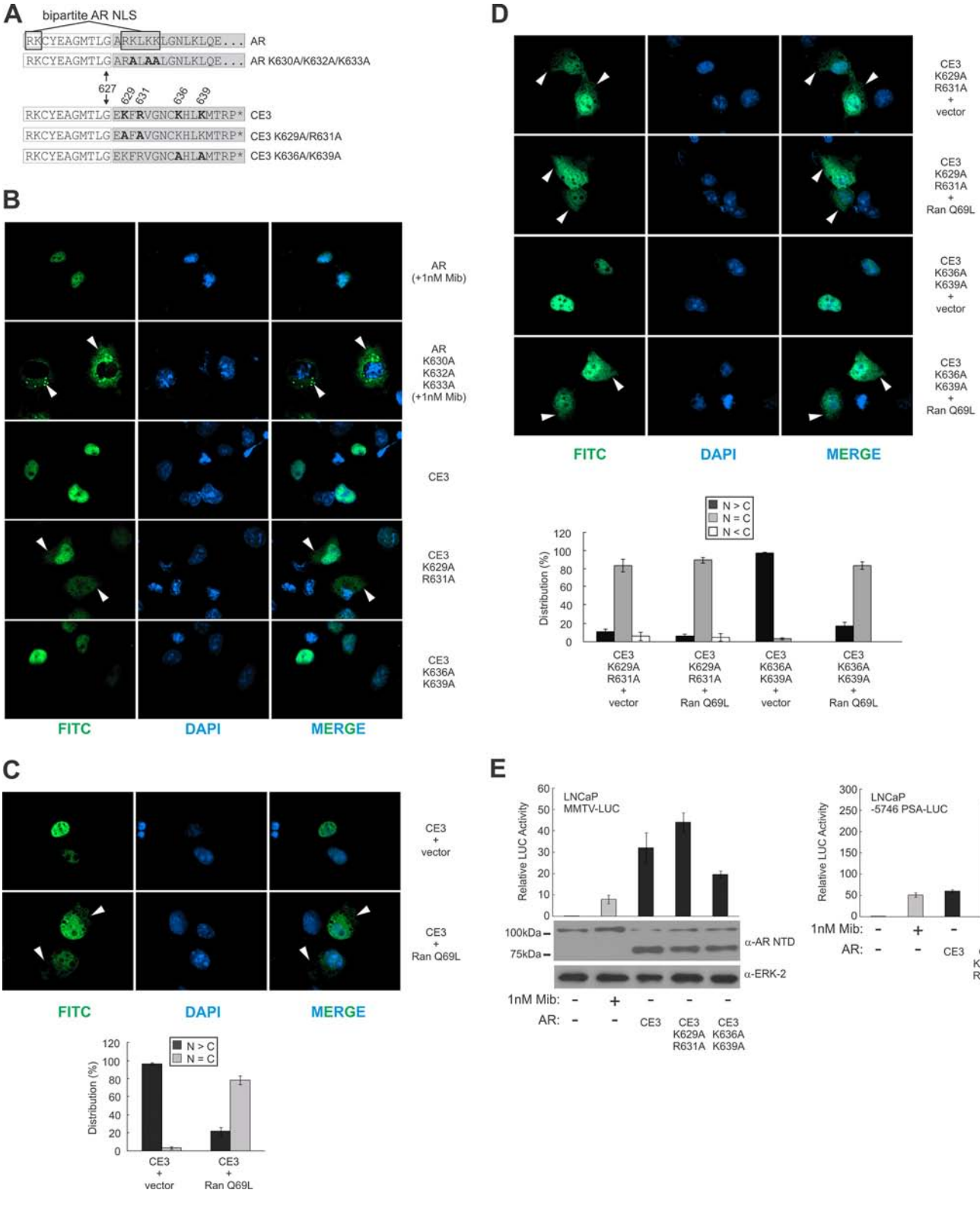
FIGURE 2. **Constitutive transcriptional activity of truncated AR variants with diverse COOH-terminal extensions.** *A*, DU145 cells were transiently transfected with 4XARE-E4-LUC or MMTV-LUC reporters and expression plasmids encoding full-length AR or truncated variants 1/2/3/CE1, 1/2/3/CE2, 1/2/3/CE3, 1/2/3/2b, and v567es. Cells were cultured in serum-free medium containing 1 nM mibolerone (*Mib*) or vehicle control (ethanol). Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent mean ± S.E. from at least three independent experiments, each performed in duplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1. *B*, LNCaP cells were transiently transfected with a −5746 PSA-LUC reporter and truncated AR variants and analyzed by luciferase assay and Western blot as in *A*. *C*, LNCaP cells were transiently transfected and analyzed exactly as described in *B* with the exception of the use of AR exon 7-targeted siRNA and siRNA-resistant AR expression constructs (denoted *sr* (17)).

microscopy (Fig. 1*B* and see supplemental Fig. 1) and biochemical fractionation (Fig. 1*C*). Truncated AR variants displayed significant differences in predominance of nuclear localization, but all were able to access the nucleus. Nuclear localization patterns were the same in AR-null Cos-7 and DU145 cells and AR-dependent LNCaP cells, indicating that nuclear localization is not affected by cell type or presence of full-length AR (Fig. 1, *B* and *C*, and see supplemental Fig. 1). To test this directly, we selectively knocked-down full-length AR expression in 22Rv1 cells using siRNA targeted to AR exon 7 and observed no effect on the nuclear localization of endogenous truncated AR variants (Fig. 1*D*). Therefore, truncated AR variants with the AR NTD/DBD core and diverse COOH-terminal extensions are nuclear proteins.

To test whether the observed differences in nuclear localization between truncated AR variants underlie differences in transcriptional activity, we performed transient transfection experiments in AR-null DU145 PCa cells and AR-dependent LNCaP cells (Fig. 2, *A* and *B*, and see supplemental Fig. 2*A*). Different truncated AR variants displayed varying strengths of transcriptional activity in a promoter-dependent but cell line-independent manner. To test a requirement for full-length AR, we performed knockdown/re-expression experiments in LNCaP cells. Knockdown of full-length AR completely inhibited ligand-dependent promoter activity, and re-expression of siRNA-resistant full-length AR restored this ligand-dependent activity. Conversely, re-expression of individual siRNA-resistant truncated AR variants was able to restore promoter activity in the absence of ligand (Fig. 2*C* and see supplemental Fig. 2*B*). The levels of transcriptional activity in these assays were nearly identical to the levels observed in the absence of knock-down (Fig. 2, *B* and *C*, and see supplemental Fig. 2), confirming that full-length AR is not required for this activity.

Reconstitution of Bipartite AR NLS Enhances Truncated AR Variant Nuclear Localization but Not Transcriptional Activity—Despite the observation that individual truncated AR variants exhibited varying degrees of transcriptional activity on AR-responsive promoters, there did not appear to be a perfect relationship between efficiency of nuclear localization and transcriptional activity. This suggests that nuclear localization is not the primary determinant of AR variant transcriptional activity. To test this concept, we focused on the AR-V7/AR3 variant generated by splicing of AR exons 1/2/3/CE3, which displayed strong nuclear localization and high transcriptional activity. This variant contains two clusters of basic amino acids in the COOH-terminal tail encoded by AR exon CE3, one of which aligns with the second basic amino acid cluster of the wild-type AR bipartite NLS (Fig. 3*A*). Consistent with previous reports (15, 23), a K630A/K632A/K633A compound mutation blocked ligand-induced nuclear localization of full-length AR (Fig. 3*B*). Alanine mutation of Lys-629 and Arg-631 in the truncated AR 1/2/3/CE3 variant, which both align to the second basic amino acid cluster in the full-length AR NLS, shifted 1/2/3/CE3 expression from predominantly nuclear to a mixed nuclear/cytoplasmic pattern (Fig. 3*B* and see supplemental Fig. 3). Conversely, alanine mutation of Lys-636 and Lys-639 in the truncated AR 1/2/3/CE3 variant had no effect on nuclear localization (Fig. 3*B*). We next asked whether dominant negative Ran Q69L, which prevents the carrier importin-β from releasing cargo into the nucleus (24), could alter subcellular distribution of the truncated AR1/2/3/CE3 variant. Indeed, dominant negative Ran Q69L caused a shift in AR 1/2/3/CE3 expression to a mixed nuclear/cytoplasmic pattern (Fig. 3*C*). A similar effect of dominant negative Ran Q69L was observed on the nuclear K636A/K639A mutant version of AR 1/2/3/CE3, but no effect was observed for the nuclear/cytoplasmic K629A/R631A mutant version, showing that the dominant negative effects of

Ran Q69L were mediated through the Lys-629/Arg-631 motif (Fig. 3*D*). These data indicate that AR 1/2/3/CE3 displays enhanced nuclear localization because amino acid residues Lys-629 and Arg-631 reconstitute the second half of the bipartite AR NLS. Notably, alanine mutations in these residues did not have the same magnitude of effect as for full-length AR, indicating alternate modes of nuclear import existed for truncated AR variants. Indeed, the K629A/R631A mutant version of AR 1/2/3/CE3 displayed a paradoxical higher level of transcriptional activity on various AR-responsive promoters in AR-dependent LNCaP cells and AR-null DU145 cells, further confirming that the classical mode of AR nuclear import is not the



main determinant of truncated AR variant activity (Fig. 3*E* and see supplemental Fig. 4).

To test this concept more rigorously, we asked whether increasing the basic amino acid content in the short COOH-terminal tail of the truncated AR 1/2/3/CE2 variant would enhance nuclear localization by reconstituting bipartite AR NLS function. Alignment of the COOH-terminal tails of AR 1/2/3/CE2 and AR 1/2/3/CE3 revealed that they both harbor arginine at position 631, but AR 1/2/3/CE2 harbors glycine at position 629 (Fig. 4*A*). As predicted, generation of a G629K mutant, which mimics the basic amino acid cluster in the AR 1/2/3/CE3 COOH-terminal tail, led to enhanced nuclear localization (Fig. 4*B*) that was reversed by dominant negative Ran Q69L (Fig. 4*C*). However, despite this enhanced nuclear localization, transcriptional activity on an AR-responsive promoter was unchanged (Fig. 4*D*).

These data indicate that truncated AR variants possess an intrinsic, basal level of nuclear localization that may be supported by a pathway(s) different from that required by full-length AR. This may be due to unmasking of additional regulatory signals or loss of the strong COOH-terminal AR nuclear export sequence by truncation of the AR LBD (25). Indeed, even nuclear localization of the AR v567es variant, which retains the AR exon 3/4 splice junction and wild-type AR NLS sequence, was only modestly affected by dominant negative Ran Q69L (see supplemental Fig. 5). We, therefore, queried whether activity of the Hsp90 chaperone complex, which is required upstream of importin-α/β for AR ligand binding and translocation to the nucleus (13), was required for nuclear access of truncated AR variants. A complete block in androgen-induced nuclear import of full-length AR was observed after treatment of cells with the Hsp90 inhibitor, 17-*N*-allylamino-17-demethoxygeldanamycin (Fig. 5*A*), but there was no effect of this inhibitor on constitutive nuclear localization of the truncated AR 1/2/3/CE3 and v567es variants (Fig. 5, *B* and *C*). These data thus confirm that the canonical pathways required for nuclear import of full-length AR are not required for nuclear import of truncated AR variants.

AR NTD Encoded by Exon 1 and AR DBD Encoded by Exons 2 and 3 Are Sufficient for Nuclear Localization and Transcriptional Activity of Truncated AR Variants—We next turned our attention to the role of the first basic amino acid cluster in the bipartite AR NLS, which is encoded by AR exon 3. Even though the AR NLS/importin-α crystal structure did not reveal a direct binding role for this part of the NLS (15), we postulated that this region may be able to compensate in the absence of the second, main cluster of basic amino acids. Therefore, we generated R617A/K618A compound mutations in full-length AR and truncated AR variants (Fig. 6*A*). Interestingly, this compound mutation did not inhibit ligand-dependent transcriptional activity or nuclear localization of full-length AR (Fig. 6, *B* and *C*), but this mutation impaired transcriptional activity of truncated AR variants (Fig. 6*D*). The R617A/K618A compound mutant version of the truncated AR 1/2/3/CE3 variant, but not the 1/2/3/CE2 variant, displayed impairment in nuclear access (Fig. 6*E* and see supplemental Fig. 6), indicating that this motif plays a minor role in truncated AR variant nuclear localization. More significantly, these data suggested that the intrinsic, basal level of nuclear localization displayed by truncated AR variants proceeds in a manner completely independent of either basic amino acid cluster in the bipartite AR NLS. To test this idea directly, we generated a R617A/K618A/K629A/R631A compound mutant of the truncated AR 1/2/3/CE3 variant to neutralize both basic amino acid clusters (Fig. 7*A*). This compound mutant version of the truncated AR 1/2/3/CE3 variant displayed roughly equal distribution in the nucleus and cytoplasm, demonstrating that basal nuclear localization of truncated AR variants could proceed independently from the canonical AR NLS (Fig. 7*B*). However, this compound mutant was transcriptionally inactive (Fig. 7, *C* and *D*), illustrating that these residues in the second α-helix of the AR DBD are important for transcriptional function.

To probe the requirement for this first cluster of basic amino acids in more detail, we generated a synthetic truncated AR variant composed of only the exon 1-encoded NTD and exon 2/3-encoded DBD (AR amino acids 1–627) as well as a R617A/K618A compound mutant version of this synthetic AR variant (Fig. 7*A*). The synthetic AR 1–627 variant displayed approximately equal expression in the nucleus and cytoplasm (Fig. 7*B*) as well as strong ligand-independent transcriptional activity on AR-responsive promoters (Fig. 7, *C* and *D*). This finding confirms that COOH-terminal tail sequences encoded by exons spliced after exon 3 are not required for transcriptional function. However, the R617A/K618A compound mutant version of the synthetic AR 1–627 variant was transcriptionally inactive (Fig. 7, *C* and *D*). This compound mutant was still able to access the nucleus, but a pattern consistent with protein aggregation was also noted (Fig. 7*B*). These data further implicate a unique and important role for exon 3-encoded Arg-617 and Lys-618 in structural integrity of the AR DBD in the context of truncated AR variants but not full-length AR.

FIGURE 3. **The COOH-terminal tail encoded by AR exon CE3 reconstitutes the canonical AR NLS.** *A*, shown is a schematic of alanine substitutions in full-length AR and the truncated AR 1/2/3/CE3 variant. *B*, Cos-7 cells expressing parental and mutant plasmids illustrated in *A* were maintained in serum-free medium containing 1 nM mibolerone (*Mib*, synthetic androgen) or ethanol (vehicle control) before staining with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). Increased cytoplasmic localization is denoted by *white arrows*. *C*, Cos-7 cells were transfected with an AR 1/2/3/CE3 expression plasmid and dominant negative Ran Q69L. Cells were stained and subjected to confocal microscopy as in *A*. Increased cytoplasmic localization is denoted by *white arrows*. Quantification of predominantly nuclear ($N > C$) *versus* equal nuclear and cytoplasmic ($n = C$) expression is shown at the *bottom*. Bars represent the mean ± S.D. of 100 cells scored in three independent experiments. *D*, Cos-7 cells were transfected with mutant plasmids indicated in *A* along with dominant-negative RanQ69L (or vector control). Cells were stained and subjected to confocal microscopy as in *A*. Increased cytoplasmic localization is denoted by *white arrows*. Quantification of predominantly nuclear ($N > C$), equal nuclear and cytoplasmic ($n = C$) or predominantly cytoplasmic ($N < C$) expression is shown at the *bottom*. *Bars* represent mean ± S.D. of 100 cells scored in three independent experiments. *E*, LNCaP cells were transiently transfected with MMTV-LUC or −5746-LUC reporters and parental and mutant plasmid constructs depicted in *A*. Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent the mean ± S.E. from at least three independent experiments, each performed in triplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1.

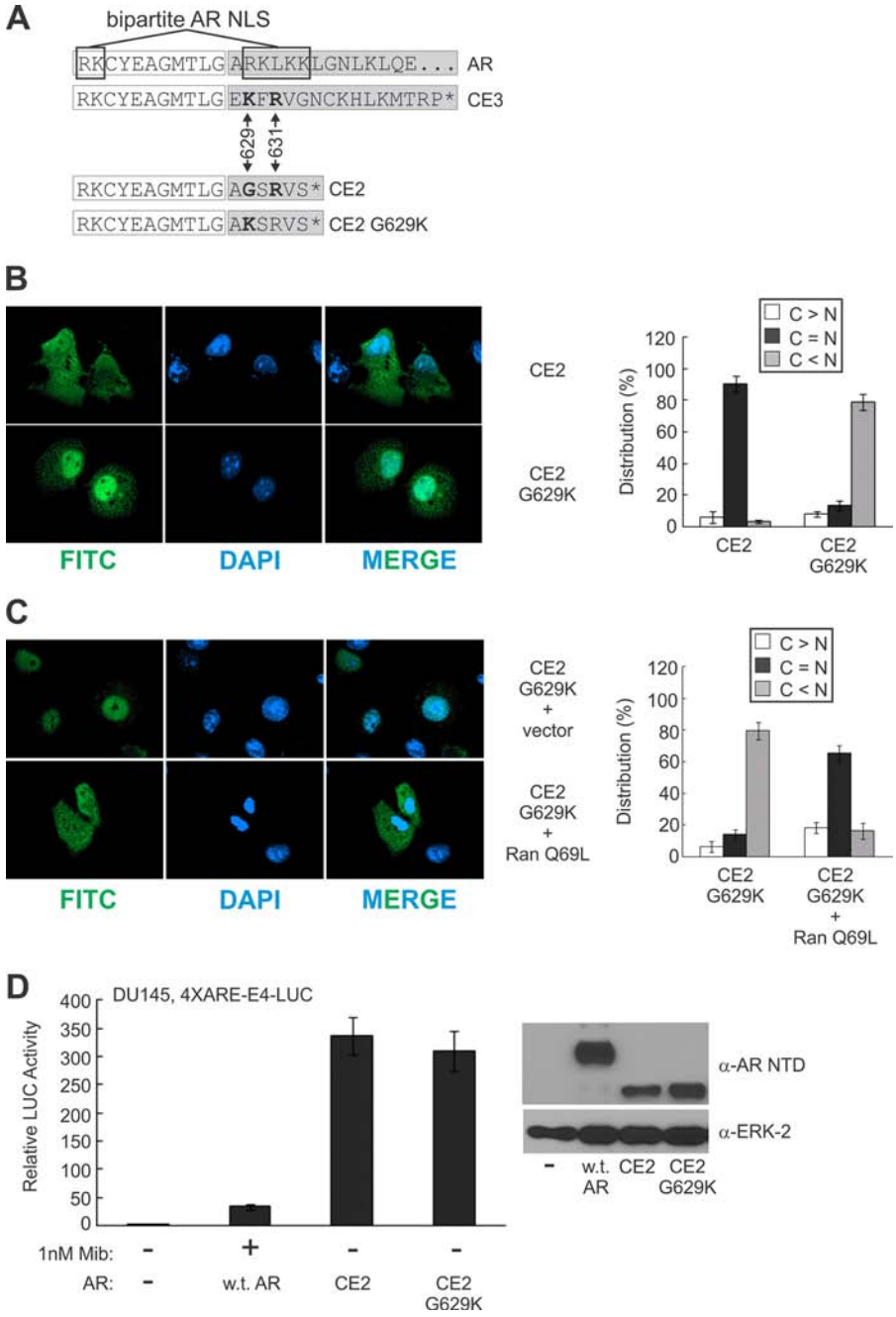


FIGURE 4. **NLS reconstitution does not enhance transcriptional activity of the truncated AR 1/2/3/CE2 variant.** *A*, shown is alignment of full-length AR, AR 1/2/3/CE3, and AR 1/2/3/CE2 and location of the G629K mutation. *B*, *left*, Cos-7 cells expressing 1/2/3/CE2 and the G629K mutant were maintained in serum-free medium and stained with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *Right*, shown is quantification of predominantly nuclear ($N > C$), equally distributed nuclear and cytoplasmic ($n = C$), or predominantly cytoplasmic ($N < C$) expression. Bars represent mean ± S.D. of 100 cells scored in 3 independent experiments. *C*, *left*, Cos-7 cells were transfected as in *B* with the addition of dominant-negative RanQ69L (or vector control). *Right*, shown is quantification of predominantly nuclear ($N > C$), equally distributed nuclear and cytoplasmic ($n = C$), or predominantly cytoplasmic ($N < C$) expression. *Bars* represent mean ± S.D. of 100 cells scored in 3 independent experiments. *D*, *left*, DU145 cells were transfected with a 4XARE-E4-LUC reporter and parental/mutant plasmid constructs depicted in *A*. *Bars* represent mean ± S.E. from at least three independent experiments, each performed in triplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1. *Right*, cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control).

AR NTD/DBD Fragment Is Sufficient for Constitutive, Ligand-independent Transcriptional Activation of AR Target Genes—To determine whether these biochemical principles applied to endogenous AR target genes, we generated lentivirus encoding GFP, AR 1/2/3/CE3, AR 1/2/3/CE3 R617A/K618A, AR 1/2/3/CE3 K629A/R631A, or the synthetic AR 1–627 variant (Fig. 8*A*). We chose to study transcriptional responses of PSA, hK2, TMPRSS2, and FKBP51 because promoters and enhancers of these genes are regulated by a variety of classical and selective androgen response elements (AREs) that may reflect differential requirements for AR COOH-terminal extensions (26). Infection of LNCaP cells with lentivirus encod-

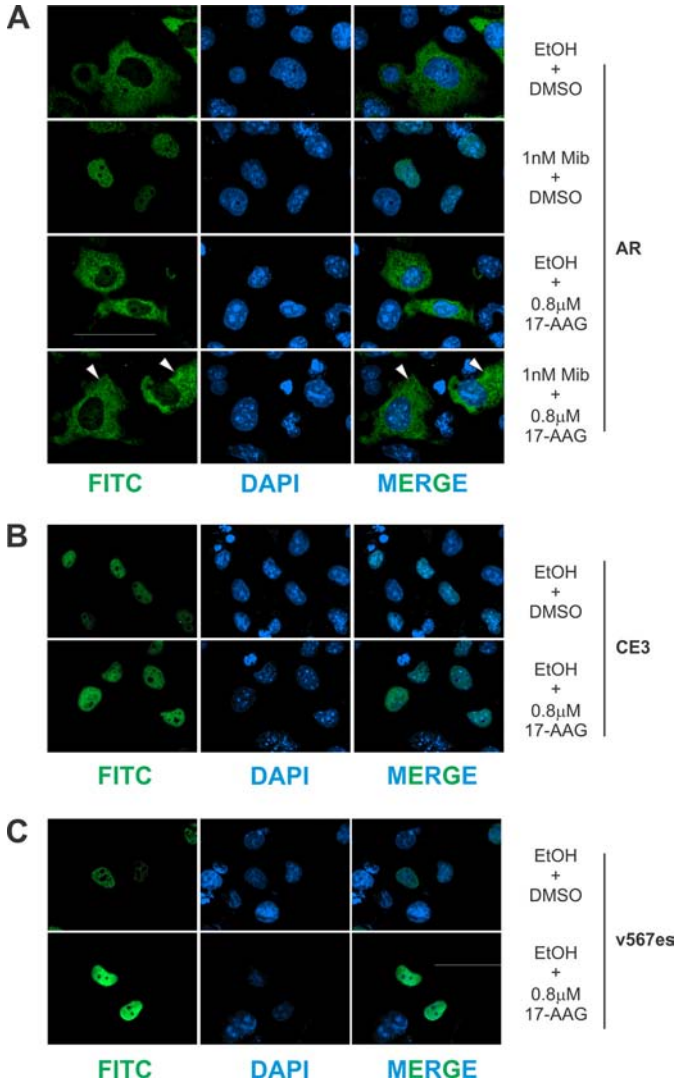


FIGURE 5. **Truncated AR variants do not require Hsp90 activity for nuclear localization.** Cos-7 cells expressing full-length AR (*A*), AR 1/2/3/CE3 (*B*), or AR v567es (*C*) were maintained in serum-free medium containing the indicated combinations of 1 nM mibolerone (*Mib*, synthetic androgen), ethanol (*EtOH*, vehicle control), 0.8 μM 17-*N*-allylamino-17-demethoxygeldanamycin (*17-AAG*, Hsp90 inhibitor), and dimetylsulfoxide (*DMSO*, vehicle control) before staining with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *White arrowheads* are shown to highlight cytoplasmic expression caused by 17-*N*-allylamino-17-demethoxygeldanamycin.

ing the truncated AR 1/2/3/CE3 variant elicited androgen-independent transcriptional activation of these AR target genes (Fig. 8*B*). Inactivation of the reconstituted AR 1/2/3/CE3 NLS by K629A/R631A mutations or truncation after position 627 still resulted in robust, ligand-independent transcriptional activation of the same AR target genes (Fig. 8*B*). However, inactivation of the Arg-617/Lys-618 motif in the second α-helix of the AR DBD of AR 1/2/3/CE3 impaired transcriptional activation of PSA, hK2, and FKBP51 target genes. Noteworthy, the TMPRSS2 gene did not display impairment in androgen-independent expression levels in response to any of these mutations in the AR DBD or COOH-terminal tail (Fig. 8*B*). Together, these data illustrate a new paradigm wherein the AR NTD/DBD core encoded by exons 1, 2, and 3 are sufficient for a basal level of access to the nucleus, engagement with endogenous AR target genes, and efficient transcriptional activation of these target genes in an androgen-free environment. Because CRPCa is characterized by persistent AR transcriptional activity under conditions of ADT, these findings indicate that AR variants consisting of the AR NTD and DBD may contribute to disease progression regardless of the identity of their COOH-terminal extensions.

AR 1/2/3/2b Protein Is Expressed in 22Rv1 Cells and Can Support Androgen-independent Growth of Prostate Cancer Cells—The CRPCa 22Rv1 cell line displays altered AR splicing patterns as a result of a 35-kb intragenic AR tandem duplication encompassing AR exon 3 (27, 28). We and others have demonstrated that 22Rv1 cells express two separate mRNAs harboring cryptic exon 2b composed of contiguously spliced AR exons 1/2/2b or AR exons 1/2/3/2b. The AR 1/2/2b variant is different from other truncated AR variants that have been reported because it encodes the NTD and first zinc finger of the AR DBD (see supplemental Fig. 7*A*). Because there is concern that many of the variant AR mRNAs that have been identified in 22Rv1 and other models may simply be spurious splicing byproducts that are degraded by nonsense-mediated RNA decay (29), we asked whether these protein species are expressed in 22Rv1 cells. To test for AR 1/2/2b expression, we first employed siRNA specific for AR exon 3, which would not target the AR 1/2/2b isoform. Transfection of 22Rv1 cells with this siRNA led to complete ablation of all truncated AR variant protein expression, indicating that AR 1/2/2b is not a protein constituent in these cells (see supplemental Fig. 7*B*). Next, we developed polyclonal antibodies specific for the COOH-terminal extension encoded by AR exon 2b. Crude antisera as well as antibodies purified on an immobilized peptide affinity column were able to immunoprecipitate an ~80-kDa AR polypeptide from 22Rv1 lysates (see supplemental Fig. 7*C*). Moreover, affinity-purified exon 2b polyclonal antibodies recognized a truncated AR variant species in Western blot experiments that was the same size as ectopic AR 1/2/3/2b protein (see supplemental Fig. 7*D*). Therefore, we conclude that AR 1/2/3/2b is expressed as a mature protein species in 22Rv1 cells.

The predominantly nuclear AR 1/2/3/CE3 variant (referred to as AR-V7 or AR3) is also expressed as a mature protein in 22Rv1 cells and can induce androgen-independent growth when expressed in androgen-dependent LNCaP cells (7, 8). In subcellular localization assays, AR 1/2/3/2b protein displayed a mixed cytoplasmic/nuclear localization pattern (Fig. 1, *B* and *C*). Therefore, we chose to employ the AR 1/2/3/2b variant to determine whether truncated AR species with partial nuclear localization could also support androgen-independent growth of PCa cells. We infected LNCaP cells with lentivirus encoding AR 1/2/3/2b, AR 1/2/3/CE3, or AR v567es and found that each of these truncated AR variants could induce an androgen-independent growth phenotype (Fig. 9*A*). These data confirm that classical NLS activity is independent from truncated AR variant function in PCa cells. Intriguingly, during the course of these studies we noted variability in the magnitude of androgen-independent growth that each AR variant induced in LNCaP cells. Paradoxically, we noted that higher levels of truncated AR variant expression consistently resulted in lower levels of

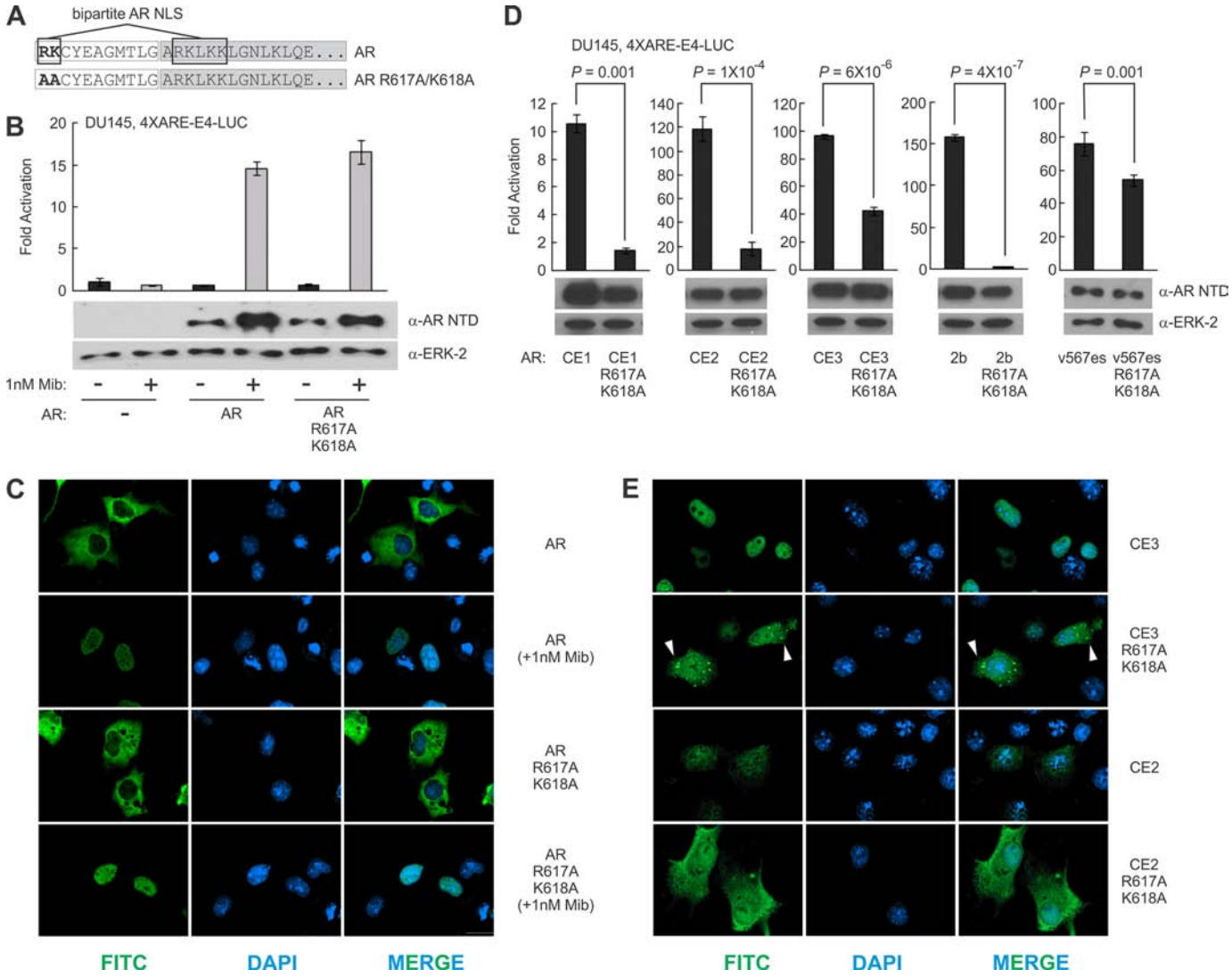


FIGURE 6. **A unique requirement for the Arg-617/Lys-618 motif in the AR DBD terminal helix.** *A*, shown is a schematic of the AR hinge region and location of the Arg-617/Lys-618 motif. *B*, DU145 cells were transiently transfected with 4XARE-E4-LUC and full-length AR or a R617A/K618A mutant. Cells were cultured in serum-free medium containing 1 nM mibolerone (*Mib*) or vehicle control (ethanol). Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent the mean ± S.E. from at least three independent experiments, each performed in triplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1. *p* values were derived using *t* tests. *C*, Cos-7 cells expressing parental or mutant plasmids illustrated in *A* were maintained in serum-free medium containing 1 nM mibolerone (*Mib*, synthetic androgen) or ethanol (vehicle control) before staining with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). Cytoplasmic localization resulting from the R617A/K618A mutation is denoted by *white arrows*. *D*, DU145 cells were transiently transfected with 4XARE-E4-LUC and parental/mutant truncated AR variants exactly as in *B*. *E*, Cos-7 cells were transfected with parental/mutant truncated AR variants, stained, and imaged exactly as in *C*. Increased cytoplasmic localization resulting from the CE3 R617A/K618A mutation is denoted by *white arrows*.

androgen-independent growth. This observation is reminiscent of the biphasic growth response of LNCaP cells wherein androgen concentrations in the 0.1–1 nM range induce proliferation, but higher doses are growth-suppressive despite robust induction of AR target genes (30–32). Indeed, LNCaP cells transduced with lentivirus encoding AR 1/2/3/CE3 displayed a dose-dependent increase in expression of PSA and TMPRSS2 but were growth-suppressed at a high expression level (Fig. 9, *B* and *C*). This growth suppression was also observed when lentivirus-infected LNCaP cells were assayed the presence of androgens (see supplemental Fig. 8*A*). Moreover, under these conditions the AR 1/2/3/CE3 variant appeared to inhibit dihydrotestosterone-induced AR transcriptional activity perhaps via competition for ARE binding or feedback inhibition of full-length AR expression (33) (see supplemental Fig. 8*B*). In similar dose-response experiments with AR 1/2/3/2b or AR v567es, androgen-independent growth of LNCaP cells was apparent at lower expression levels, but higher levels of expression blocked this effect (see supplemental Fig. 8, *C* and *D*). Together, these data indicate that truncated AR variants can support androgen-independent PCa cell growth regardless of nuclear localization efficiency. To test this we performed AR knockdown/re-expression experiments in the 22Rv1 cell line (Fig. 9, *D* and *E*). Knocking-down AR expression using an exon 1-targeted siRNA inhibited androgen-independent growth, which was fully restored by lentiviral re-expression of siRNA-resistant AR 1/2/3/CE3 (Fig. 9, *D* and *E*). The nuclear/cytoplasmic AR 1/2/3/CE3 K629A/R631A mutant was also able to effectively

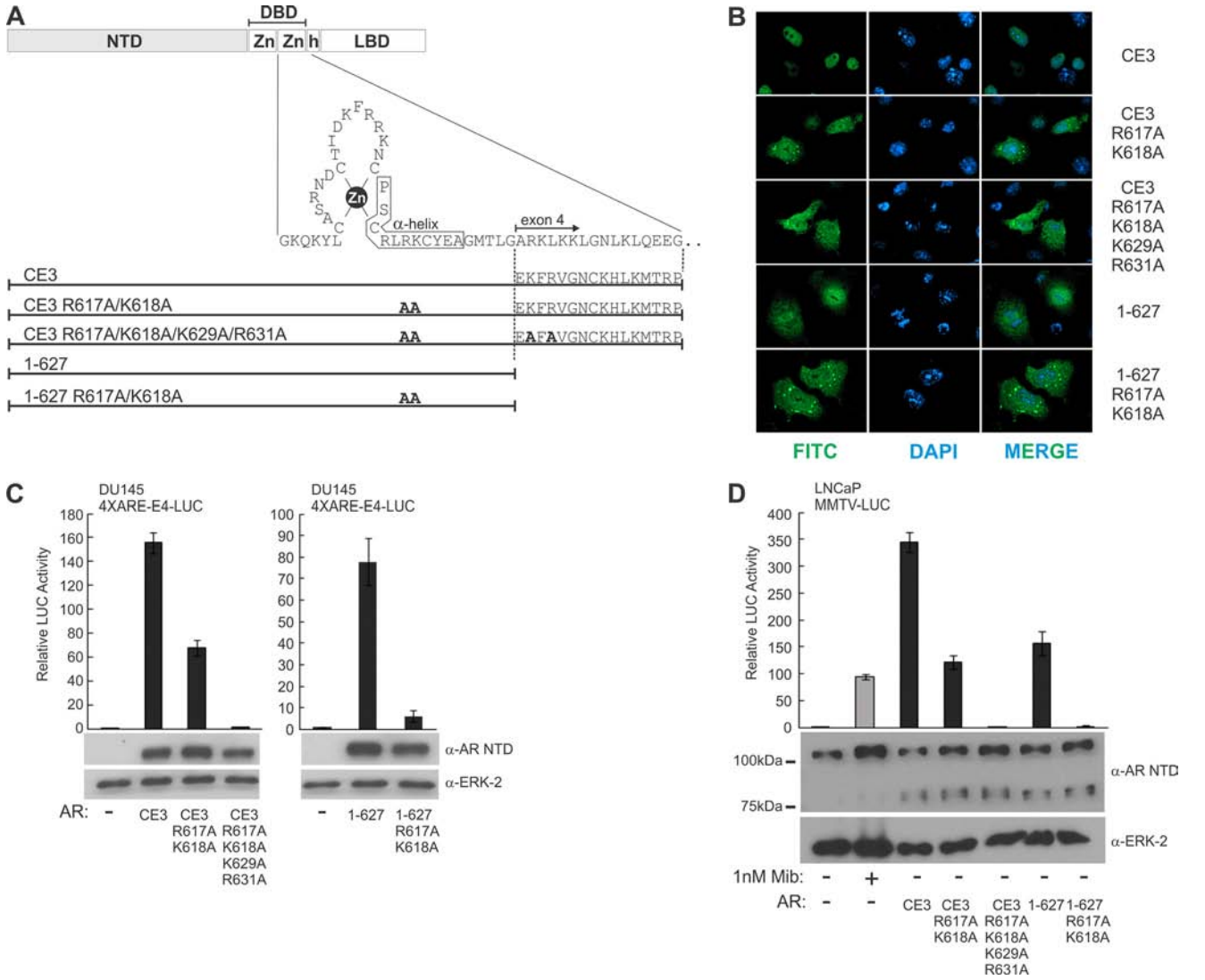


FIGURE 7. **The AR NTD/DBD core is sufficient for nuclear localization and transcriptional activity of truncated AR variants.** *A*, shown is a schematic of COOH-terminal deletions and alanine substitution mutations relative to key structural motifs in the second zinc finger of the AR DBD and hinge region. Zinc finger and α-helix locations were adapted from a prior depiction of the AR DBD crystal structure (46). *B*, Cos-7 cells expressing constructs depicted in *A* were maintained in serum-free medium and stained with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *C*, DU145 cells were transiently transfected with 4XARE-E4-LUC and constructs depicted in *A*. Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent mean ± S.E. from at least three independent experiments, each performed in triplicate. *D*, LNCaP cells were transiently transfected with MMTV-LUC and constructs depicted in *A*. Luciferase activity and transgene expression were assessed exactly as described in *C*. *Mib*, mibolerone.

restore androgen-independent growth, but the AR 1/2/3/CE3 R617A/K618A mutant displayed impairment (Fig. 9, *D* and *E*), which is consistent with the effects of these mutations on truncated AR variant transcriptional activity (Fig. 8, *A* and *B*).

In summary, our data demonstrate that a subset of truncated AR variants display enhanced nuclear localization due to reconstitution of classical NLS activity. However, a high, basal level of nuclear localization is mediated by the AR NTD/DBD core encoded by AR exons 1, 2, and 3. This basal level of nuclear localization is sufficient for strong, androgen-independent transcriptional activation of AR target genes and induction of androgen-independent growth. Therefore, truncated AR variants with diverse COOH-terminal extensions can contribute to CRPCa pathology by driving persistent AR activity during ADT.

DISCUSSION

Steroid receptors have extensive similarities in DBD and LBD structure, and a wealth of mechanistic information exists for steps leading to transcriptional activation. A key requirement for transcriptional activity of steroid receptors is interaction of unliganded receptors with the Hsp90 chaperone machinery. This interaction occurs exclusively through the LBD via direct binding to Hsp40 and Hsp70 (13, 34, 35). The Hsp90 chaperone complex association maintains AR in a conformation that inhibits DNA binding (36) and has high affinity for androgens (37). Chaperone release from steroid receptors is ligand-dependent, which results in a conformational change that exposes the bipartite AR NLS (RKCYEAGMTLGARKLKK) in the flexible hinge region between the AR DBD and

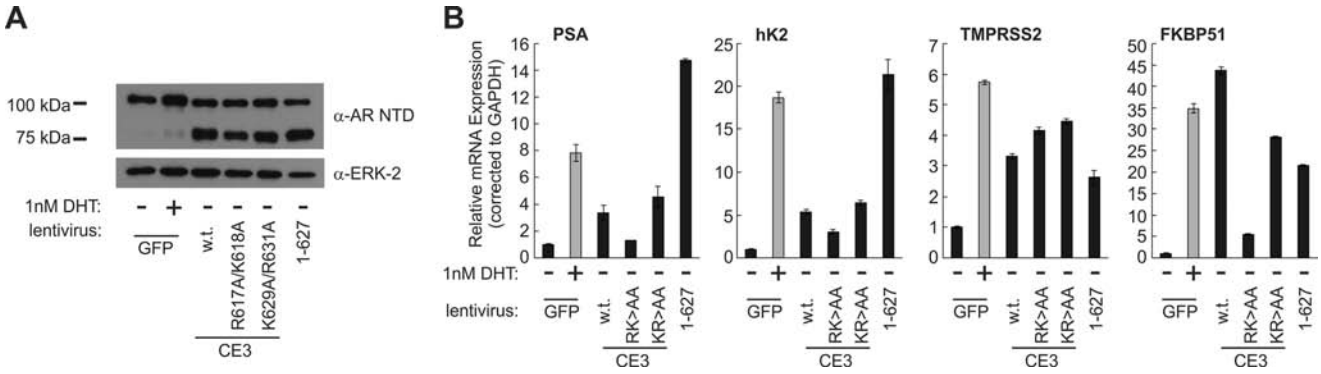


FIGURE 8. **The AR NTD/DBD core is sufficient for transcriptional activation of endogenous AR target genes.** *A*, LNCaP cells were transduced with lentivirus encoding GFP, parental and mutant versions of AR 1/2/3/CE3, or AR 1–627. Transduced cells were maintained under serum-free conditions and stimulated with 1 nM dihydrotestosterone (*DHT*) or vehicle control (ethanol) for 24 h before Western blot analysis with antibodies specific for the AR NTD or ERK-2 (loading control). *B*, LNCaP cells were transduced exactly as in *A*, and RNA was subjected to quantitative RT-PCR using primer sets specific for GAPDH, PSA, hK2, TMPRSS2, and FKBP51. Expression is shown relative to GAPDH as determined using the formula $2^{-\Delta\Delta Ct}$. *Bars* represent the mean ± S.D. from a triplicate experiment representative of three biological replicates.

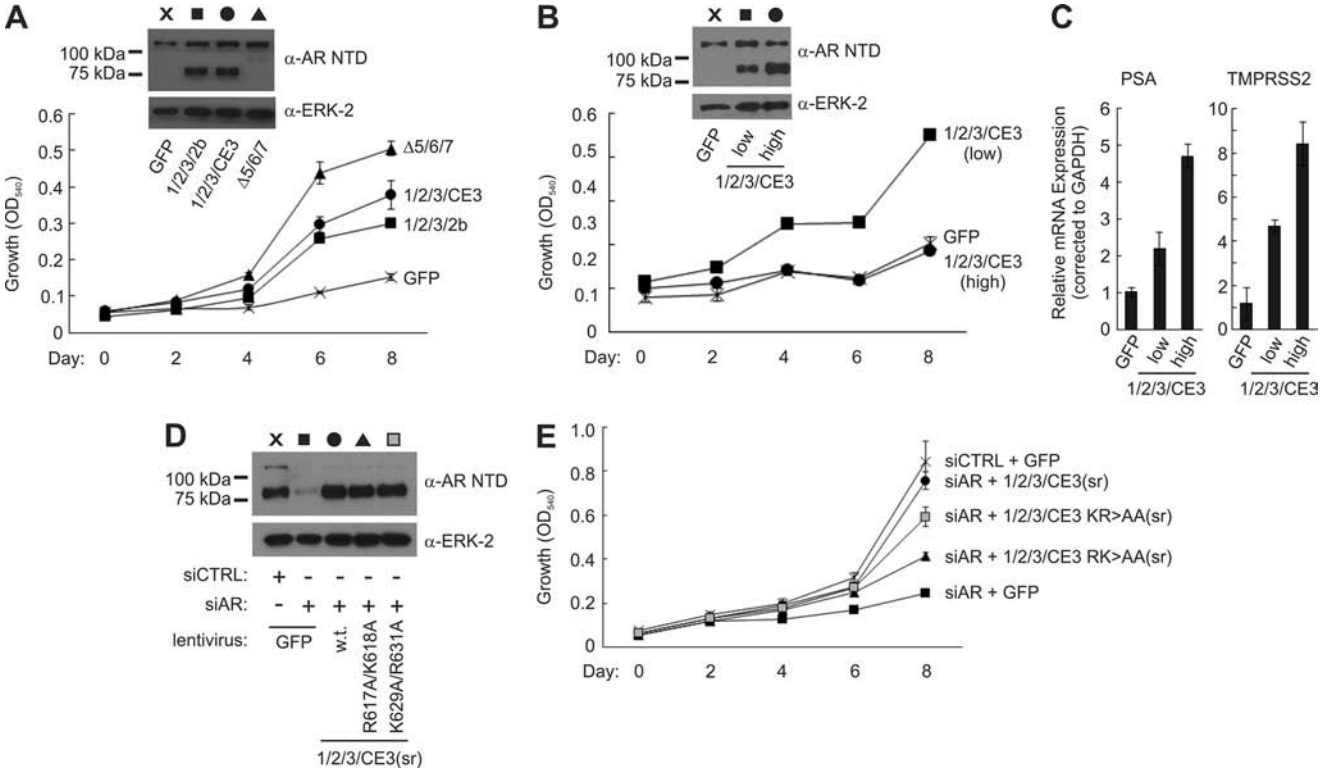


FIGURE 9. **Truncated AR variants support biphasic, androgen-independent growth of PCa cells.** *A*, LNCaP cells transduced with lentivirus encoding AR 1/2/3/2b, AR 1/2/3/CE3, or AR v567es were maintained in medium containing 10% charcoal-stripped (steroid-depleted) serum (10% charcoal-stripped serum). *Inset*, transduced cell lysates were analyzed by Western blot with antibodies specific for the AR NTD or ERK-2 (loading control). *Graph*, transduced cells were seeded on 24-well plates in medium containing 10% charcoal-stripped serum. At the indicated time points, cells were fixed and stained with crystal violet. Intensity of crystal violet staining (A_{540}) was used as a surrogate of cell number. Data represent the mean ± S.D. from a quadruplicate experiment representative of three biological replicates. *B*, LNCaP cells were transduced with increasing doses of lentivirus encoding AR 1/2/3/CE3 and subjected to Western blot (*inset*) and growth assay (*graph*) exactly as in *A*. *C*, LNCaP cells transduced as in *B* were subjected to quantitative RT-PCR using primer sets specific for GAPDH, PSA, and TMPRSS2. Expression is shown relative to GAPDH as determined using the formula $2^{-\Delta\Delta Ct}$. *Bars* represent the mean ± S.D. from a triplicate experiment representative of three biological replicates. *D*, 22Rv1 cells were transduced with lentivirus encoding GFP or siRNA-resistant forms (denoted *sr* (17)) of AR 1/2/3/CE3, AR 1/2/3/CE3 R617A/K618A, or AR 1/2/3/CE3 K629A/R631A and electroporated with siRNA targeted to AR exon 1 as indicated. Transduced/electroporated cells were subjected to Western blot with antibodies specific for the AR NTD or ERK-2 (loading control). *E*, 22Rv1 cells transduced/electroporated as in *D* were seeded on 24-well plates in medium containing 10% charcoal-stripped serum. At the indicated time points, cells were fixed and stained with crystal violet. Intensity of crystal violet staining (A_{540}) was used as a surrogate of cell number. Data represent the mean ± S.D. from a quadruplicate experiment representative of two biological replicates.

LBD. The crystal structure of the AR NLS peptide in complex with importin-α has revealed that the second cluster of basic amino acids (RKLKK), which is encoded by AR exon 4, engages with the major NLS binding site in importin-α (15). Interestingly, data presented here demonstrate that truncated AR variants display a constitutive, basal level of nuclear localization sufficient for ligand-independent transcriptional activity regardless of whether they harbor the exon 4-encoded NLS or NLS-like COOH-terminal extensions. Moreover, we have demonstrated that truncated AR variants access the nucleus inde-

pendently of the Hsp90 chaperone complex. Therefore, the signals that are critical for the regulatory cycle of the full-length AR appear to be dispensable for truncated AR variants.

It is well established that removal of the AR LBD results in ligand-independent AR transcriptional activity mediated by the AR NTD, but these early studies involved the use of constructs with deletions after the hinge region to retain the complete AR NLS (22, 38). The straightforward idea that NLS-mediated nuclear localization and transcriptional activity are intertwined has also led to controversy over the significance of many truncated AR variants that have been identified to date. For example, it has been postulated that truncated AR variants retaining the exon 4-encoded AR NLS would be functional transcription factors, whereas others would be transcriptionally incompetent (9, 10). This concept was supported by the demonstration that individual truncated AR variants display varying degrees of transcriptional activity (7) and an apparent correlation between the extent of nuclear localization and transcriptional activation (10). However, the current study demonstrates that transcriptional strength of individual truncated AR variants is a promoter-dependent phenomenon and independent of the magnitude of subcellular localization. Indeed, point mutations that inhibited the constitutive nuclear localization of the AR 1/2/3/CE3 isoform did not inhibit transcriptional activity. Similarly, point mutations that enhanced the nuclear localization of the AR 1/2/3/CE2 variant did not increase transcriptional activity. Even a synthetic truncated AR variant (1–627) that completely lacked the importin-α binding site (15) was transcriptionally active in a ligand-independent manner. Because these mechanistic principles were independent of promoter or cell line and were also extended to clinically relevant endogenous AR target genes, we conclude that constitutive transcriptional activity is likely an inherent property of truncated AR variants retaining the NTD/DBD core encoded by AR exons 1, 2, and 3. Our data also demonstrate that truncated AR variants with this NTD/DBD core can support androgen-independent growth when expressed in PCa cells. However, we have also shown that individual truncated AR variants need to be evaluated for growth-promoting effects very cautiously, especially in LNCaP cells, because the constitutive, androgen-independent transcriptional activity appears to induce a biphasic growth response in a fashion similar to high-dose androgen treatment (30–32). The molecular basis for the biphasic androgen effect is largely unknown but may arise from AR-mediated activation of differentiation-promoting genes or AR-mediated transcriptional inhibition of the AR promoter at higher androgen concentrations (33).

If the canonical nuclear localization signal is dispensable for truncated AR variant function, then what are the mechanisms that promote their entry into the nucleus? A previous live cell imaging study with fluorescence-labeled AR fragments demonstrated that the AR NTD has strong nuclear import activity in isolation (39). Moreover, deletion of the RKLKK motif in full-length AR has been shown to impair nuclear localization but induces a paradoxical superactivity in response to androgens (40), which may be due to enhanced intranuclear mobility (41). However, studies where the AR NLS has been manipulated by mutation and/or deletion have historically been difficult to interpret because lysine residues in the AR NLS are also acetylation targets (42, 43). The fact that the AR 1–627 fragment employed in this study could access the nucleus and activate endogenous AR target genes with high efficiency clearly confirms that functional nuclear targeting signals exist outside of the AR hinge/LBD region. Based on these findings, we conclude that the canonical NLS in the AR hinge region is not the only determinant of AR nuclear access and transcriptional activity in PCa cells.

Intriguingly, a previous study demonstrated that androgen-independent growth induced by truncated AR variants required full-length AR (10). One explanation for this phenomenon could be a physical interaction between full-length AR and truncated AR variants, which is supported by *in vitro* experiments with overexpressed full-length AR and AR v567es (9). However, we have recently demonstrated that the LuCaP 86.2 xenograft, where the AR v567es variant was discovered, harbors a 8.5-kb deletion of AR exons 5, 6, and 7 in the sole AR gene copy at Xq11–12 (44). This would preclude a scenario where full-length AR and AR v567es could co-exist in the same cell in this model. Similarly, in this and other studies we have demonstrated that knockdown of full-length AR in the 22Rv1 and CWR-R1 models of CRPCa has no effect on androgen-independent expression of AR target genes or androgen-independent growth. Conversely, knock-down of endogenously expressed truncated AR variants inhibits both of these parameters (28, 44). These data strongly support the concept that truncated AR variants possess the biochemical properties required to independently support ongoing PCa cell growth during ADT. Indeed, PCa cells with AR intragenic rearrangements and altered splicing patterns have a competitive growth advantage under castrate conditions (28, 44). The finding that intragenic copy number imbalances are frequent in CRPCa (44) portends complex and diverse AR splicing patterns in human tumors, which is also supported by unbiased tiling array studies (27). Antibody development efforts in this and previous studies show that rearrangement-dependent splicing alterations do indeed give rise to translated, functional proteins such as AR 1/2/3/2b and AR 1/2/3/CE3 (7, 8). Our data establish new biochemical principles that will be critical for studying the role of known as well as yet-to-be-discovered truncated AR variant species in PCa progression.

Our data also highlight the potential application of targeting truncated AR variants for therapy of CRPCa. For example, inhibitors such as EPI-001, a bisphenol A-derivative that appears to interfere with the function of transcriptional activation domains in the AR NTD (45), would be anticipated to block transcriptional activity and growth-supporting functions of truncated AR variants. Our data also show that truncated AR variants have a unique requirement for structural integrity of the second α-helix in the AR DBD, which might be an additional focal point for inhibitory strategies. We have further shown that higher levels of overexpression of the truncated AR 1/2/3/CE3 variant in LNCaP cells leads to paradoxical lower levels of cell growth, especially under conditions where androgens are present. Androgen-induced expression of AR target genes was also reduced after expression of the truncated AR 1/2/3/CE3 variant. This indicates that targeted inhibition of

truncated AR variants may be most effective when activity of full-length AR is durably suppressed. However, it is also possible that these results may reflect competition between ligand-bound AR and truncated AR variants for ARE binding and/or an acute sensitivity of LNCaP cells to excessive levels of AR signaling. Further investigation in additional models is required to fully understand the potential application and optimal conditions for targeting truncated AR variants in CRPCa.

In summary, our data demonstrate that truncated AR variants with the NTD/DBD core are constitutively active, ligand-independent transcription factors that can support androgen-independent growth of PCa cells. Expression of truncated AR variants is frequent in CRPCa metastases and associated with poor prognosis (11). Therefore, AR variant species with the NTD/DBD core should be viewed as key therapeutic targets at this stage of the disease.

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